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PRINCIPAL INVESTIGATOR: Carolyn L. Smith, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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6. AUTHOR(S)
Carolyn L. Smith, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-MAIL:
carolyns@bcm.tmc.edu

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13. ABSTRACT *(Maximum 200 Words)*

There are two known receptors for estrogens, ERα and ERβ. The existence of ERβ was only recently appreciated, and little is understood about its ability to be activated by intracellular signaling pathways in the absence of estrogens. The purpose of this research program is to characterize the ability of ERβ to by activated by various ligand-independent signaling pathways, and to characterize the structural regions of ERβ, in comparison to ERα, that regulate how this receptor isotype responds to intracellular cross-talk. We have found that stimulation of HeLa cells with forskolin and IBMX results in the activation of ERα and ERβ dependent expression in a receptor-dependent and promoter context-dependent manner, and that protein kinase A mediates this response. Factors that interact with an AP-1 binding site contribute to forskolin/IBMX activation of estrogen receptor-dependent gene expression, and do so in a manner that does not require the A/B domain of either receptor. ERα and ERβ differ in their requirement for interaction with the putative AP-1 binding site factor. ERβ-mediated transcription is dependent on this site suggesting that forskolin activation reflects the synergistic action between ERβ and other transcription factor, while ERα can activate gene expression in a AP-1-independent manner.

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Introduction

Breast cancer remains the major cancer (excluding skin cancer) among women in the United States with more than 180,000 new cases anticipated in 2000. The requirement of estrogens for normal breast development is well documented (1). However, estrogens also have been linked to breast cancer, presumably through their ability to stimulate cell proliferation (2) and inhibition of estrogen action therefore has been a primary objective in the treatment, and more recently the prevention of, breast cancer. For many years, estrogen effects were thought to be mediated by a unique, high affinity intracellular receptor protein, the estrogen receptor (ER), that is a member of a superfamily of transcription factors (3,4). The basic mechanisms of ER activity have been ascertained. Hormone binding to ER results in receptor homodimerization and binding to specific enhancer DNA elements located in the promoter regions of target genes (5,6). This process, which is accompanied by increases in ER phosphorylation (7-12), enables "activated" receptors to regulate the transcription of hormone-responsive target genes and the resulting changes in mRNA and protein synthesis are ultimately responsible for alterations in cellular function. The structural features of the estrogen receptor (ERα) responsible for hormone binding, dimerization, DNA binding and transcriptional activation have been identified (3,13-16) and these studies have provided the basis of our understanding of the molecular mechanisms by which estrogens regulate the growth and differentiation of mammary tissues.

Clearly, the transcriptional activity of the ER can be regulated by estrogens, such as 17β-estradiol (E2). However, the ERα also can be activated in the absence of exogenous ligand by agents that stimulate intracellular signal transduction cascades (EGF, IGF-1, heregulin, dopamine, TPA and cAMP) (7,17-23) or inhibit protein phosphatases (okadaic acid) (19). Furthermore, cyclin D1, independent of cyclin-dependent kinases, also can activate the ER in the absence of estrogen (24). The ERα knock-out mouse model confirms that ERα is required for some but not all *in vivo* EGF effects and established the importance of ligand-independent activation of ER to physiological events (25). Most of these ligand-independent activation pathways (with the exception of cyclin D1) increase receptor phosphorylation (7,12,23,26,27) and mutation of the only known ligand-independent (EGF) phosphorylation site (serine^{118}) to an alanine residue abolishes EGF activation of the ER (28), suggesting that phosphorylation may play an important role in these activation pathways. However, this point mutant does not block cAMP-mediated gene expression and different domains are required to respond to EGF and cAMP signaling pathways (29), suggesting that multiple mechanisms must exist to enable ER to activate target gene expression in response to diverse regulatory events.

In 1996, a new member of the nuclear receptor superfamily was cloned from a prostate cDNA library (30). When the resulting cDNA was sequenced and expressed, it became apparent that a novel estrogen receptor had been identified. This new member of the nuclear receptor superfamily was named ERβ, and the original estrogen receptor was renamed ERα. The ERβ binds to estradiol with an affinity (K_d 0.4 nM) similar to ERα and binds to the same DNA response element as ERα (30-32). Thus, it is reasonable to predict that ERβ regulates the expression of at least a subset of ERα target genes. However, the relatively undeveloped mammary glands in the ERα knock-out mouse indicate that ERβ is not equivalent to ERα (33). The reasons for this are unclear, but could be related to differential expression and/or differences in the ability of α and β estrogen receptors to activate target gene expression. Mouse, rat and human ERβs are approximately 65 amino acids smaller than their corresponding α-receptors,

and the A/B, D and F domains (**Figure 1**) are poorly conserved (30,32,34). Furthermore, the ligand binding domains (domain E) of ERα and ERβ are only ~55% identical and rat ERα and ERβ receptors do not bind equally well to all ligands (31). The expression patterns of ERα and ERβ mRNAs are different but overlapping (31) suggesting that the genes for ERα and ERβ are independently regulated. However, both ERα and ERβ mRNA have been detected in human mammary gland, breast tumors and several human breast cancer cell lines (35,36). Taken together, these data suggest that ERβ is likely to play a role in mediating estrogen action in mammary gland, but that this receptor is unlikely to be functionally equivalent to ERα.

The identification of a second estrogen receptor raised a number of important biological questions such as, what is the expression of ERβ, relative to ERα, in normal and malignant mammary tissue? However, knowledge regarding the expression of ERβ in mammary gland will be of limited value without detailed information on the transcriptional activity of ERβ. Do ERα and ERβ respond similarly to ligand-independent pathways? Are currently used antiestrogens equally effective antagonists of ERα and ERβ? Do ERα and ERβ activate the same target genes to a comparable extent? Studies of this nature will provide the information necessary to determine whether resources are required to develop new strategies to more effectively and/or selectively block ERα- and ERβ-mediated estrogen effects. Indeed, increasing evidence demonstrates that ERα and ERβ are not functionally equivalent, and our experiments reported below contribute to the foundation upon which new strategies to regulate ERα and ERβ biological activity can be developed. Moreover, comparing and contrasting the structure/function relationships of ERα and ERβ with respect to activation by ligand-independent pathways represents a novel approach to study mechanistic questions relating to activation of gene expression in the absence of estrogens.

Body

A new member of the nuclear receptor superfamily, ERβ, has been identified that binds to estrogens with high affinity, and binds to the same DNA response elements as the classical estrogen receptor, ERα. Both of these ligand-regulatable transcription factors possess a well-defined, centrally located, DNA binding domain and carboxy-terminal domain, which contains a ligand-dependent activation function (AF-2); however the amino terminus which possesses a second activation function (AF-1) is poorly conserved. Thus, it is highly likely that that the biological activity of ERβ will differ from that of ERα. This hypothesis is being tested in the following two technical objectives:

1. To determine if estrogen-independent signaling pathways can stimulate ERβ transcriptional activity.
2. To determine what regions of ERβ contribute to its estrogen-independent transcriptional activity and to compare these regions to known ERα activation functions to characterize the structural features of these receptors that contribute to their respective biological properties.

The originally reported form of ERβ represented a truncated version of the subsequently identified full length form of the receptor. In the first year of this award, we conducted experiments directed towards resolving differences in activity and expression of the full-length

and truncated forms of ERβ. All of our studies in year 2 utilized the full-length form of ERβ, (unless deletion mutations were being analyzed).

We have continued our analyses of the ability of ERβ to be activated by ligand-independent, cAMP stimulated, signaling pathways. As reported last year, both ERα and ERβ are activated in cells treated with forskolin and isobutylmethylxanthine (IBMX). Forskolin is an activator of adenylyl cyclase and IBMX is a phosphodiesterase inhibitor, and treatment of cells with these compounds therefore results in an increase in intracellular cAMP levels. In transient transfection assays, 10 μM forskolin and 100 μM IBMX stimulated the ERβ activation of ERE-Elb-CAT target gene by ~6-fold while ERα-dependent gene expression was stimulated by ~3-fold (**Figure 2**). Minimal change in target gene expression was observed in cells transfected with the reporter gene and an empty expression vector indicating that the increased CAT activity is receptor-dependent (**Figure 2**). Furthermore, an ERα mutant possessing point mutations in its DNA binding domain (C201H/C205H) was unable to mediate forskolin/IBMX-induced CAT gene expression indicating that receptor binding to DNA was required (**Figure 2**). Further analysis demonstrates that a target gene lacking the ERE cannot be stimulated by the forskolin/IBMX-induced signaling pathway in cells expressing ERα or ERβ (**Figure 3**). Thus, intracellular cAMP signaling pathways have the potential to activate the transcriptional activity of both ERα and ERβ, and this activation is dependent on the expression of an estrogen receptor, the receptor's ability to bind to DNA and the presence of an estrogen response element within the target gene. To ensure that target gene expression resulted from forskolin/IBMX activation of the cAMP-dependent/protein kinase A (PKA) signaling pathway and not a non-specific event, we assessed whether the specific protein kinase inhibitor, H89, could block activation of ERα- and ERβ-dependent transcription. As shown in **Figure 4**, H89 blocked forskolin activation of both receptor isotypes, but not transcriptional activity stimulated by E2, supporting that activation occurs via a cAMP/PKA dependent signaling pathway.

The preceding experiments were performed with the ERE-E1b-CAT or ERE-tk-CAT target genes which consist of an estrogen response element linked to a TATA box or thymidine kinase promoter, and the CAT reporter gene. To investigate whether the target gene influenced the ability of forskolin/IBMX to activate receptor-dependent gene expression, the same experiment was repeated, but using other target genes. As can be seen in **Figure 5** (and data not shown), the expression of the pS2-CAT, pATC0, pATC1, pATC2, ERE-E1b-Luc and pC3-Luc target genes was not stimulated by forskolin/IBMX, although E2 increased gene expression in every target gene that possessed an ERE. In contrast, the activity of the ERE-tk-CAT, ERE-Elb-CAT (Figures 2 & 3) and pC3110-tk-Luc (data not shown) target genes was increased. This indicates that the ability of the cAMP-dependent PKA pathway to activate target gene expression was dependent on the nature of the reporter gene examined. The majority of these target genes contain consensus EREs, so we therefore turned our attention to other potential transcription factor binding sites that are present within the synthetic target gene vectors, in order to determine what role, if any, they play in forskolin/IBMX activation of ER-dependent gene expression.

Many vectors have an imperfect AP-1 binding site (also known as a TPA responsive element or TRE) located several hundred base pairs upstream of their minimal promoters. Both the ERE-E1b-CAT and ERE-tk-CAT vectors have such a site. In order to determine if this binding site contributed to the overall activation of gene expression following forskolin/IBMX stimulation of cells, we made a four nucleotide insertion within the putative AP-1-binding site of ERE-Elb-

CAT that prevents AP-1 from binding to DNA (37). Interestingly, forskolin/IBMX was unable to activate ERβ-dependent expression of the resulting mutated target gene even though E2 could still stimulate ERβ activity (**Figure 6**). In contrast, mutation of the putative AP-1 site (mTRE) did not block forskolin/IBMX activation of target gene activity by ERα, although it decreased the relative magnitude of the response. Similar results were obtained when the AP-1 site was removed (ΔNdeI-Eco0109) through a more extensive deletion of 195 bp surrounding the AP-1 binding site (**Figure 6**). These experiments suggested that AP-1 sites were contributing to the ability of ERα and ERβ to stimulate ERE-dependent gene expression, and that factors that bound to the AP-1 binding and ERE sites were cooperating to bring about activation of transcription. In support of this, we (**Figure 7**) and others (38,39) have shown that forskolin/IBMX activates AP-1-dependent gene expression of target genes that lack estrogen response elements. Although the reporter genes that we have used in our studies are by their very nature, synthetic in origin, it is interesting to note that AP-1 binding sites are widely distributed in the promoter region of many endogenous genes, including the progesterone receptor which has been shown to be stimulated in an ER-dependent manner by treatment of cells with cholera toxin and IBMX (40), an agent that like forskolin/IBMX will stimulate intracellular cAMP production/accumulation and activation of a PKA signaling pathway, or forskolin (41).

It has been reported previously that ERα can interact with c-jun, one component of the AP-1 transcription factor directly through the receptor's A/B domain (42). However, both ERα and c-jun are also able to bind to coactivators, such as CBP/p300 (43-45), and it is possible that the cooperative functional interactions between these two transcription factors are direct (e.g. they bind to one another) or indirect (e.g. they interact via association with a common coativator). To begin to distinguish between these two possibilities, we analyzed the ability of ERα and ERβ deletion mutants lacking their A/B domains to be activated by the forskolin/IBMX-stimulated signal transduction pathway. In our first experiment, the A/B domains of ERα and ERβ were deleted to generate expression vectors for ERα-179C and ERβ-143C, respectively. These deletion mutants were tested for their ability to activate the expression of the ERE-Elb-CAT target gene in response to the forskolin/IBMX-induced signaling pathway. Forskolin/IBMX activated the transcriptional activity of ERα-179C and ERβ-143C, the former to an extent reduced in comparison to its respective wild type receptor (**Figure 8, top**). However, when assays were performed with the mutated target gene, ERE-E1b-CAT (mTRE) in which the AP-1 site has been disrupted by a four nucleotide insertion within the putative AP-1 site, neither receptor deletion mutant was able to stimulate transcription of the target gene (**Figure 8, bottom**). Taken together, this indicates that activation of target gene transcription by the cAMP-dependent/PKA signal transduction pathway requires an estrogen receptor as well as another transcription factor activity, in this case AP-1, and that these interactions do not require the A/B domain of either ERα or ERβ. Ongoing experiments are further assessing whether the interaction between either ER and the putative AP-1 transcription factor is direct (e.g. jun interaction with ER) or indirect (e.g. jun interaction with ER via a bridging coactivator such as SRC-1 or CBP). It should also be noted that these experiments highlight a functional difference between ERα and ERβ. While both of these receptors, when lacking their A/B/ domains can stimulate gene expression of the ERE-E1b-CAT target gene, only full-length ERα retains the ability to activate transcription of the mutated TRE-containing target gene [ERE-E1b-CAT (mTRE)], while full-length ERβ is unable to do so. This suggests that ERα has an enhanced

ability to interact with cellular proteins that increase its transcriptional potency. We have evidence of this from other experiments we have conducted (see below).

Work performed in the first year of this project resulted in the construction of vectors that will enable us to examine the structural features that enable ERα and ERβ to respond in equal or dissimilar ways to various estrogen-independent signals. Expression vectors for chimeric proteins consisting of the amino-terminal domains of ERα and ERβ (individually), have been fused *C*-terminal to the heterologous Gal4 DNA binding domain (DBD). These constructs isolate the A/B region that encompasses the AF-1 domain from the remainder of the respective receptor and will facilitate an examination of their transcriptional activity in response to ligand-independent signaling pathways. When assessed in HeLa cells, the A/B domain of ERα has significantly more transcriptional activity than the Gal4 DBD alone, while the A/B domain of ERβ has weak activity in comparison to ERα, but is distinguishable from that observed for the GAL4 DBD alone. We have examined these chimeric proteins for their ability to be activated by forskolin/IBMX-stimulated signal transduction pathways and found that the activity of neither Gal-A/Bα nor Gal-A/Bβ was affected (data not shown). However, it should be noted that these experiments were performed on the pG5-Luc reporter construct that does not possess an AP-1 binding site in a position comparable to that found in the ERE-E1b-CAT expression vector. These experiments will therefore be repeated using a target gene, 4x17mer-tk-CAT which does so. Expression vectors for chimeric ERα and ERβ proteins in which the respective ligand binding domains have been fused *C*-terminal to the Gal4 DBD have also been generated in the last year. These constructs have been tested for their ability to be stimulated by estradiol, and are appropriately regulated (data not shown). They will now be used to assess the ability of forskolin and other signaling pathways to stimulate estrogen receptor-dependent transcription. Collectively, these constructs will enable us to compare and contrast the ability of various estrogen-independent signaling pathways to activate a) the full-length receptor, b) the A/B region (AF1), c) the DNA and ligand binding domain and d) the ligand binding domain alone (AF2).

Last year we reported that the intrinsic transcriptional activities of the A/B domains of ERα and ERβ were distinct, with the activity of ERβ being approximately 33% of the corresponding ERα domain. Since coactivator proteins are thought to be major determinants of the transcriptional activity of transcription factor activation domains, we wanted to examine whether the A/B domain of ERα and ERβ differed in their ability to be coactivated by SRC-1, a member of the p160 family of steroid receptor coactivators (46), or SRA, a recently identified novel coactivator of the steroid receptor family that appears to exert its biological effects as a RNA molecule (47). While SRC-1 exerts its effects via the AF-1 and AF-2 domains of steroid receptors, SRA appears to mediate its biological effects via the AF-1 domain of steroid receptors. In HeLa cells transiently transfected with an expression vector for SRC-1, both Gal-A/Bα and Gal-A/Bβ chimeras were coactivated by SRC-1, in agreement with other investigators. In contrast, SRA was able to coactivate the transcriptional activity of only the A/B domain of ERα, but not ERβ (**Figure 9**). Thus these two AF-1 domains have a differential ability to functionally interact with coactivator proteins, and this may explain, in part, the relative differences in the ability of these two domains to stimulate target gene expression. Furthermore, differences such as these may also contribute to the differential ability of steroid receptors to respond to intracellular signaling pathways as we have observed in our studies of forskolin activation of ERα and ERβ. These differences will be further investigated in the coming year in order to determine whether differences in the ability of these coactivators to bind to the A/B domains of

ERα and ERβ play a significant role relative to their ability to activate transcription in response to ligand-independent signaling pathways.

Key Research Accomplishments

1. Stimulation of HeLa cells with forskolin and IBMX results in the activation of ERα and ERβ dependent expression of the ERE-E1b-CAT and ERE-tk-CAT synthetic target genes.
2. Activation of target gene expression is receptor-dependent and requires the receptor to bind to its DNA response element.
3. Forskolin/IBMX stimulated estrogen receptor activation is mediated by a protein kinase A signaling pathway.
4. Forskolin/IBMX stimulation of estrogen receptor-dependent gene expression is promoter-dependent.
5. Factors that interact with an AP-1 binding site contribute to forskolin/IBMX activation of estrogen receptor-dependent gene expression.
6. The A/B domain is not required for forskolin/IBMX activation of estrogen receptor-dependent gene expression.
7. ERα and ERβ differ in their requirement of the putative AP-1 binding site factor for their ability to be activated by a forskolin/IBMX activated signaling pathway.
8. Forskolin/IBMX signal pathways do not alter the transcriptional activity of the ERα or ERβ A/B domains.
9. The A/B domains of ERα and ERβ differ in their ability to be stimulated by the SRA coactivator.

Reportable Outcomes

A portion of the work outlined in this progress report was presented at the Keystone Symposia – Nuclear Receptor 2000, Steamboat Springs, CO. (March 25-31, 2000) in poster form. The abstract (see Appendix) was entitled "Stimulation of estrogen receptor α and β transcriptional activity by the RNA coactivator, SRA". This work has been submitted for publication as a portion of a manuscript entitled "SRA coactivates the AF-1 domain of ERα but not ERβ, and stimulates the agonist activity of the partial antiestrogen, 4-hydroxytamoxifen" (see Appendix).

Conclusions

The originally published amino acid sequence of ERβ represents an amino-terminally truncated form, which lacks the first 45 amino acids of this receptor isotype. In transient transfection assays, ERα is clearly more active than the long and short forms of ERβ. The

potentially large differences in $ER\beta_S$ and $ER\beta_L$ expression levels indicate that their relative expression levels must be taken into account when considering transactivation activity. Furthermore, the AF-1 activity of ERα exceeds that of ERβ and this likely contributes to the relative differences in transcriptional activity observed for these two receptor isotypes.

Both isotypes of estrogen receptor (α and β) can be activated in the absence of exogenous estrogens. In cells treated with forskolin and IBMX, ERα and ERβ were activated by a cAMP signaling pathway. This indicates that there is sufficient homology between these two receptor isotypes to mediate activation of gene expression by this signaling pathway. This activation pathway required the expression of estrogen receptors within the target cell, the presence of an estrogen response element in the target gene, and that the receptor can bind to DNA. However, the stimulation of receptor-dependent transcription can be significantly enhanced by the presence of the binding site for another transcription factor, in these studies a putative AP-1 binding site. Furthermore, the ability of ERα and ERβ to participate in this combinatorial response differs, supporting our original hypothesis that the ability of both estrogen receptor isotypes needs to be examined in order to determine the potential of each of these receptors to respond to ligand-independent signaling pathways

As anticipated, the experiments performed to date have provided information on the transcriptional activity of ERβ relative to ERα, as well as the ability of ERβ to respond to an alternative signaling pathway, induced by elevated intracellular cAMP, in the absence of estrogens. Taken together, this information will increase our understanding of the molecular mechanisms by which ERα and ERβ respond to cross-talk within a cell. It also will provide a framework for critical evaluation of whether it is possible to selectively regulate ERα and ERβ transcriptional activity.

References

1. Cullen KJ, Lippman ME 1989 Estrogen Regulation of Protein Synthesis and Cell Growth in Human Breast Cancer. Vitamins and Hormones 45:127-172.
2. Engel LW, Young NA 1978 Human breast carcinoma cells in continuous culture: A review. Cancer Res 38:4327-4339.
3. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889-895.
4. Tsai M-J, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Ann Rev Biochem 63:451-486.
5. Tsai SY, Carlstedt-Duke J, Weigel NL, Dahlman K, Gustafsson J-A, Tsai M-J, O'Malley BW 1988 Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. Cell 55:361-369.
6. Kumar V, Chambon P 1988 The estrogen receptor binds tightly to its response element as a ligand-induced homodimer. Cell 55:145-156.
7. Aronica SM, Katzenellenbogen BS 1993 Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-1. Mol Endocrinol 7:743-752.
8. Denton RR, Koszewski NJ, Notides AC 1992 Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. J Biol Chem 267(11):7263-7268.
9. Washburn TF, Hocutt A, Brautigan DL, Korach KS 1991 Uterine estrogen receptor in vivo: phosphorylation of nuclear specific forms on serine residues. Mol Endocrinol 5:235-242.
10. Ali S, Metzger D, Bornert JM, Chambon P 1993 Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. EMBO J 12(3):1153-1160.
11. Arnold SF, Obourn JD, Jaffe H, Notides AC 1994 Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. Mol Endocrinol 8:1208-1214.
12. Goff PL, Montano MM, Schodin DJ, Katzenellenbogen BS 1994 Phosphorylation of human estrogen receptor: Identification of hormone-regulated sites and examination of their influence on transcriptional activity. J Biol Chem 269:4458-4466.
13. Kumar V, Green S, Stack G, Berry M, Jin J-R, Chambon P 1987 Functional domains of the human estrogen receptor. Cell 51:941-951.
14. Webster NJG, Green S, Jin J-R, Chambon P 1988 The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. Cell 54:199-207.

15. Webster NJG, Green S, Tasset D, Ponglikitmongkol M, Chambon P 1989 The transcriptional activation function located in the hormone-binding domain of the human oestrogen receptor is not encoded in a single exon. EMBO J 8:1441-1446.

16. Fawell SE, Lees JA, White R, Parker MG 1990 Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. Cell 60:953-962.

17. Smith CL, Conneely OM, O'Malley BW 1993 Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone. Proc Natl Acad Sci USA 90:6120-6124.

18. Ignar-Trowbridge DM, Pimentel M, Parker MG, McLachlan JA, Korach KS 1996 Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. Endocrinology 137:1735-1744.

19. Power RF, Mani SK, Codina J, Conneely OM, O'Malley BW 1991 Dopaminergic and ligand-independent activation of steroid hormone receptors. Science 254:1636-1639.

20. Newton CJ, Buric R, Trapp T, Brockmeier S, Pagotto U, Stalla GK 1994 The unliganded estrogen receptor (ER) transduces growth factor signals. J Steroid Biochem Molec Biol 48:481-486.

21. Ma ZQ, Santagati S, Patrone C, Pollio G, Vegeto E, Maggi A 1994 Insulin-like growth factors activate estrogen receptor to control the growth and differentiation of the human neuroblastoma cell line SK-ER3. Mol Endocrinol 8:910-918.

22. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA 1993 Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol 7:992-998.

23. Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, Gorman CM, Parker MG, Sliwkowski MX, Slamon DJ 1995 HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene 10:2435-2446.

24. Zwijsen RML, Wientjens E, Klompmaker R, van der Sman J, Bernards R, Michalides RJAM 1997 CDK-independent activation of estrogen receptor by cyclin D1. Cell 88:405-415.

25. Curtis SW, Washburn TF, Sewall C, DiAugustine R, Lindzey J, Couse JF, Korach K 1996 Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. Proc Natl Acad Sci USA 93:12626-12630.

26. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270:1491-1494.

27. Joel PB, Traish AM, Lannigan DA 1995 Estradiol and phorbol ester cause phosphorylation of serine 118 in the human estrogen receptor. Mol Endocrinol 9:1041-1052.

28. Bunone G, Briand P-A, Miksicek RJ, Picard D 1996 Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. The EMBO Journal 15:2174-2183.

29. El-Tanani M, Green CD 1997 Two separate machanisms for ligand-independent activation of the estrogen receptor. Mol Endocrinol 11:928-937.

30. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925-5930.

31. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson J 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138:863-870.

32. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V 1997 Cloning, chromosomal localization and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353-365.

33. Korach KS, Couse JF, Curtis SW, Washburn TF, Linzey J, Kimbro KS, Eddy EM, Migliaccio S, Snedeker SM, Lubahn DB, Schomberg DW, Smith EP 1996 Estrogen receptor gene disruption molecular characterization and experimental and clinical phenotypes. Recent Progress in Hormone Research 51:159-188.

34. Mosselman S, Polman J, Dijkema R 1996 ERb: identification and characterization of a novel human estrogen receptor. FEBS Letters 392:49-53.

35. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantx S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson J-A 1997 Human estrogen receptor β-gene structure, chromosomal localization and expression pattern. J Clin Endocrinol Metab 82:4258-4265.

36. Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC, Watson PH 1998 Estrogen receptor-β mRNA variants in human and murine tissues. Mol and Cell Endocrin 138:199-203.

37. Jonat G, Rahmsdorf HJ, Park KK, Cato ACB, Gebel S, Ponta H, Herrlich P 1990 Antitumor promotion and antiinflammation: Down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell 62:1189-1204.

38. de Groot RP, Sassone-Corsi P 1992 Activation of Jun/AP-1 by protein kinase A. Oncogene 7:2281-2286.

39. Kvanta A, Fredholm BB 1993 Synergistic effects between protein kinase C and cAMP on activator protein-1 activity and differentiation of PC-12 pheochromocytoma cells. J Mol Neurosci 4:205-214.

40. Aronica SM, Katzenellenbogen BS 1991 Progesterone receptor regulation in uterine cells: stimulation by estrogen, cyclic adenosine 3',5'-monophosphate, and insulin-like growth factor I and suppression by antiestrogens and protein kinase inhibitors. Endocrinol 128:2045-2052.

41. Sumida C, Pasqualini JR 1990 Stimulation of progesterone receptors by phorbol ester and cyclic AMP in fetal uterine cells in culture. Mol Cell Endocrinol 69:207-215.

42. Webb P, Lopez GN, Uht RM, Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 9:443-456.

43. Hanstein B, Eckner R, DiRenzo J, Halachmi S, Liu H, Searcy B, Kurokawa R, Brown M 1996 p300 is a component of an estrogen receptor coactivator complex. Proc Natl Acad Sci USA 93:11540-11545.

44. Arias J, Alberts AS, Brindle P, Claret FX, Smeal T, Karin M, Feramisco J, Montminy M 1994 Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. Nature 370:226-229.

45. Bannister AJ, Oehler T, Wilhelm D, Angel P, Kouzarides T 1995 Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro. Oncogene 11:2509-2514.

46. Onate SA, Tsai SY, Tsai M-J, O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354-1357.

47. Lanz RB, McKenna NJ, Onate SA, Albrecht U, Wong J, Tsai SY, Tsai M-J, O'Malley BW 1999 A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. Cell 97:17-27.

Appendices

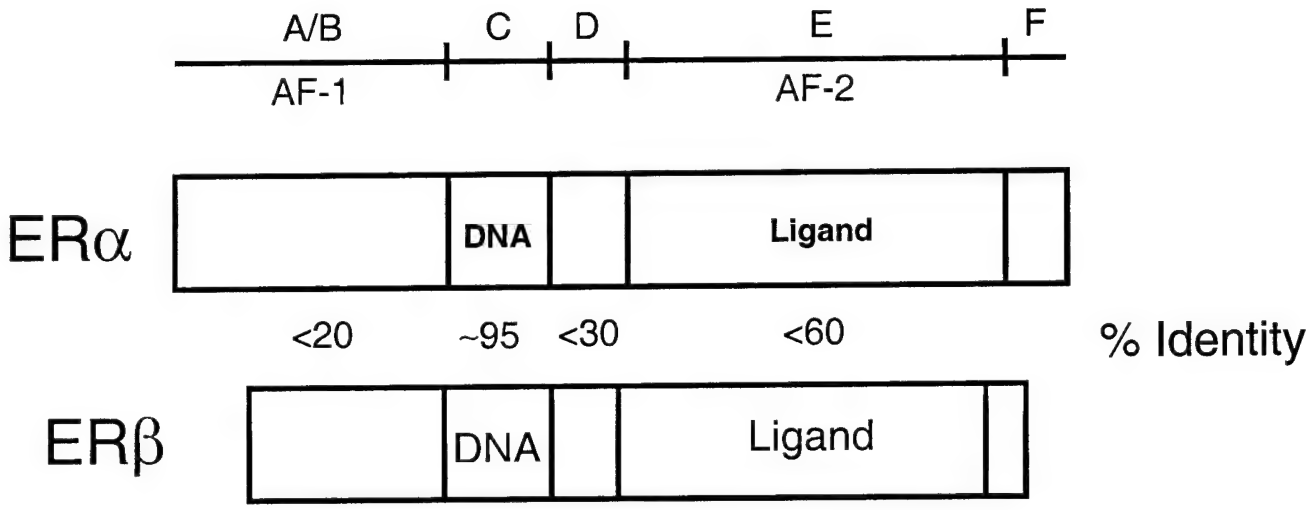

Figure 1: Structure of ERα and ERβ. The *top panel* represents the location of various regions of estrogen receptor (A to F) and its activation functions (AF-1 and AF-2). The bottom panel represents the comparative structure of ERα and ERβ. Values given between the two receptor forms represent approximately homologies in their respective amino acid sequences.

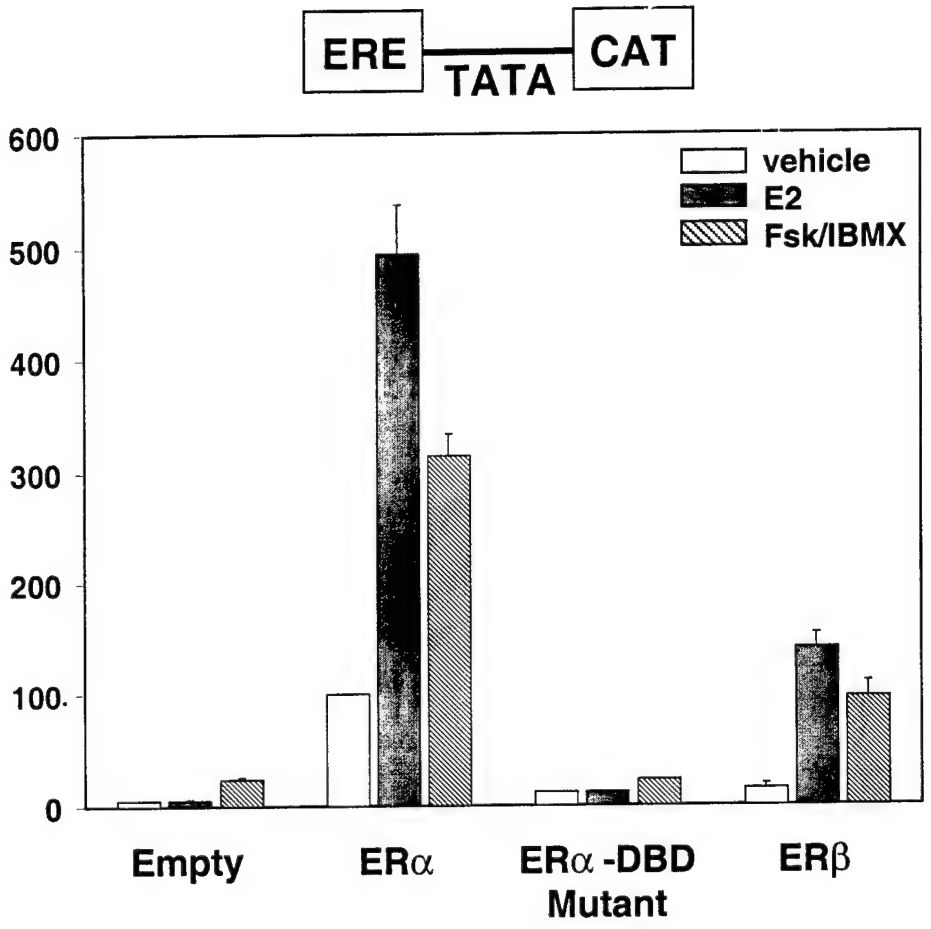

Figure 2: ERα is More Active than ERβ in Response to Stimulation with Forskolin and IBMX. HeLa cells were cotransfected with 1 μg ERE-E1b-CAT reporter plasmid and 10 ng $pCMV_5$, $pCMV_5$-hERα (ERα), pCR3.1-hERα-C201/205H (ERα-DBD Mutant) or $pCXN_2$-hERβ (ERβ) and stimulated with 1nM E2 or 10μM forskolin + 100 μM IBMX. CAT measurements were corrected for protein values and standardized to ERα activity in the presence of ethanol. Transfections were performed in duplicate and values represent the mean ± SEM of at least three experiments.

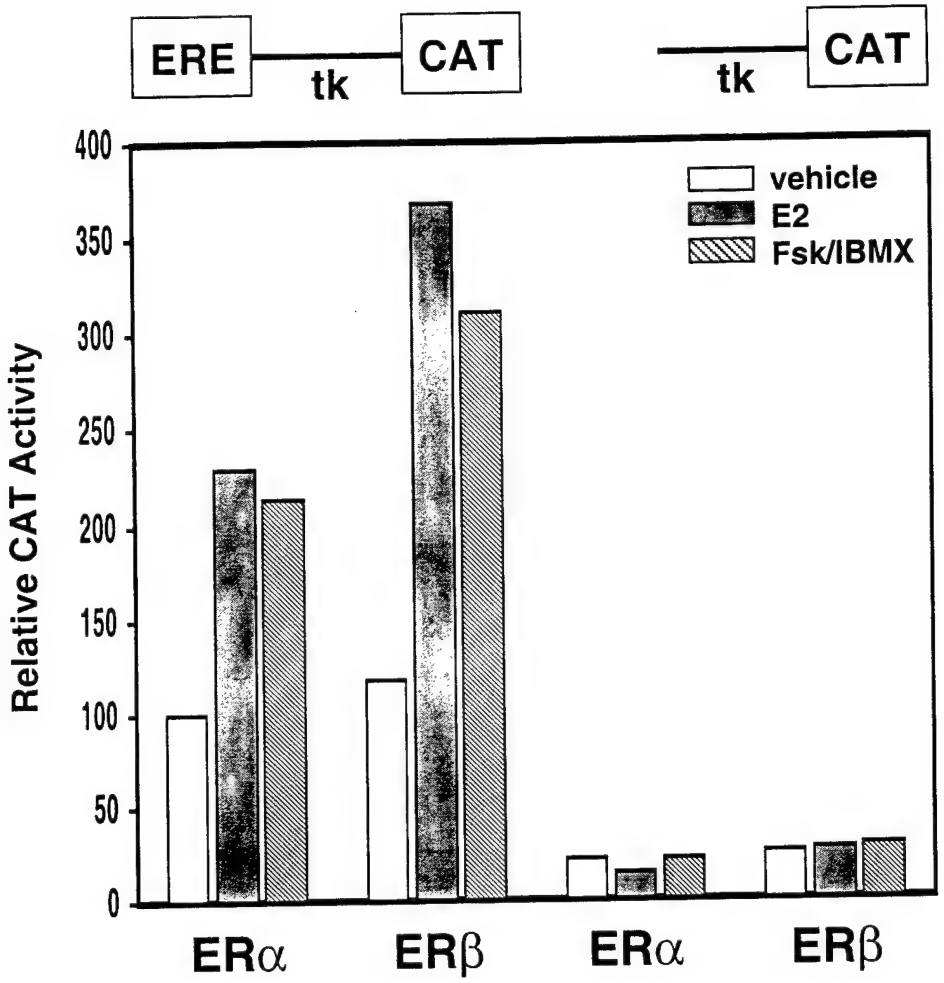

Figure 3: Estrogen Response Element is Needed for ERα and ERβ activation by Forskolin. HeLa cells were cotransfected with either 1 μg ERE-tk-CAT or tk-CAT reporter plasmid along with 10 ng $pCMV_5$-hERα (ERα) or $pCXN_2$-hERβ (ERβ) and stimulated with 1nM E2 or 10μM forskolin + 100 μM IBMX. CAT measurements were corrected for protein values and standardized to ERα activity on the ERE-tk-CAT reporter in the presence of ethanol. Transfections were performed in duplicate and values represent the mean of two experiments.

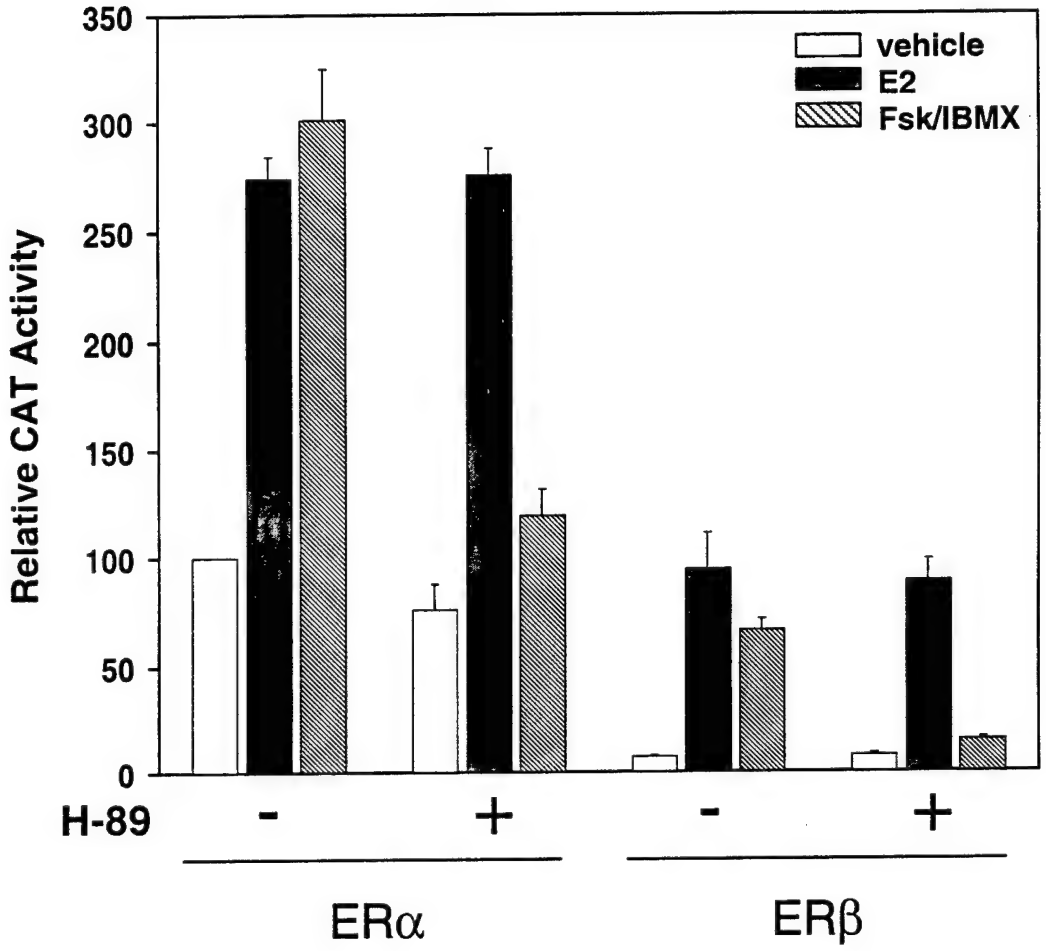

Figure 4: Forskolin Activation of ERα and ERβ is Mediated by Protein Kinase A. HeLa cells were cotransfected with 1 μg ERE-E1b-CAT reporter plasmid and 10 ng $pCMV_5$-hERα (ERα) or $pCXN_2$-hERβ (ERβ). Cells were stimulated with either 1nM E2 or 10μM forskolin + 100 μM IBMX in the presence or absence of 10 μM H89 inhibitor. CAT measurements were corrected for protein values and standardized to ERα activity on the ERE-E1b-CAT reporter in the presence of ethanol. Transfections were performed in duplicate and values represent the mean ± SEM of at least three experiments.

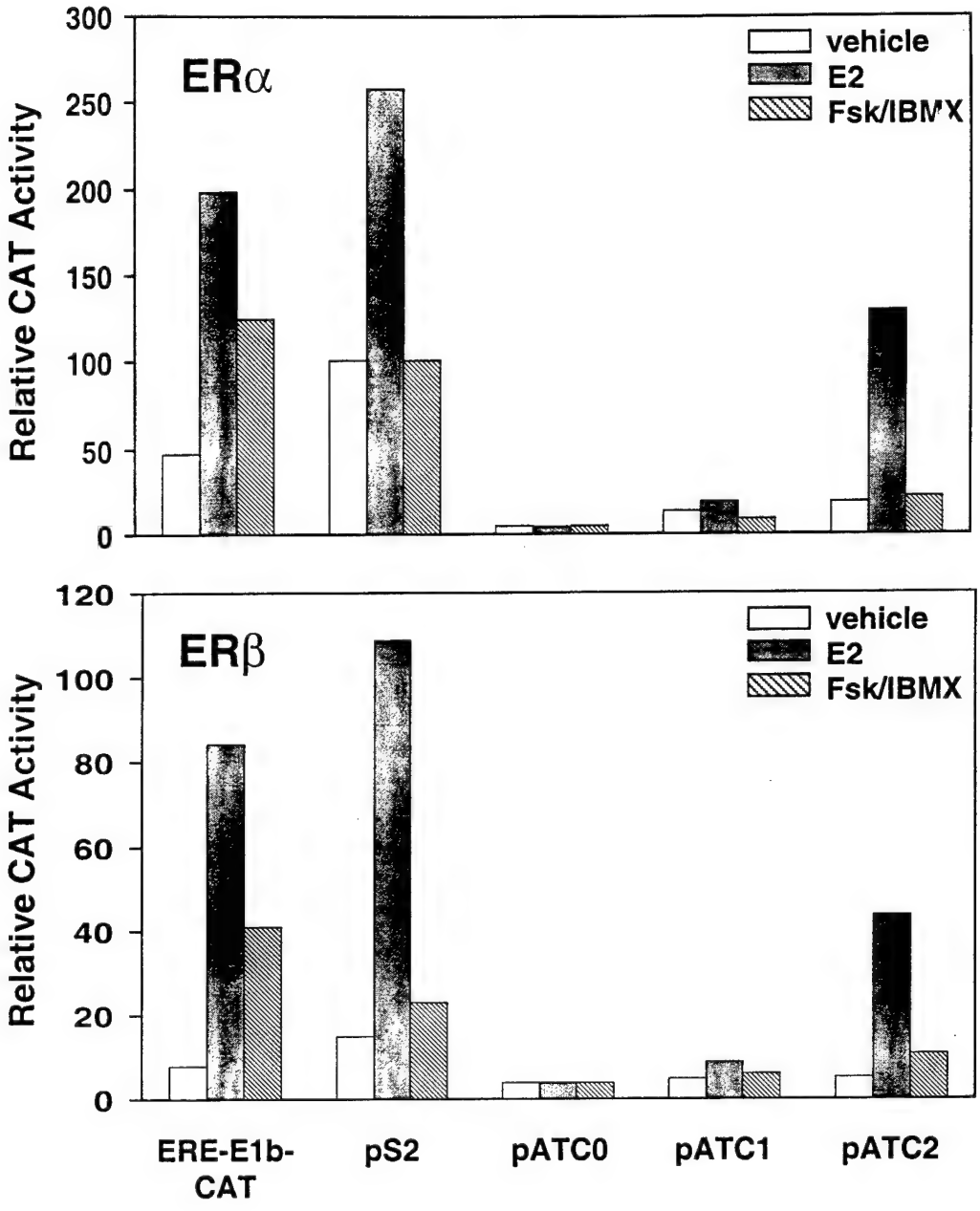

Figure 5: PKA-induced Activation of ERα and ERβ is Promoter Context Dependent. HeLa cells were cotransfected with either 1 μg ERE-E1b-CAT, pS2-CAT, pATC0, pATC1, or pATC2 reporter plasmid and 10 ng $pCMV_5$-hERα (ERα) or $pCXN_2$-hERβ (ERβ). Cells were stimulated 1nM E2, or 10μM forskolin + 100 μM IBMX. CAT measurements were corrected for protein values and standardized to ERα activity on the ERE-E1b-CAT reporter in the presence of ethanol. Transfections were performed in duplicate and values represent the mean of two experiments.

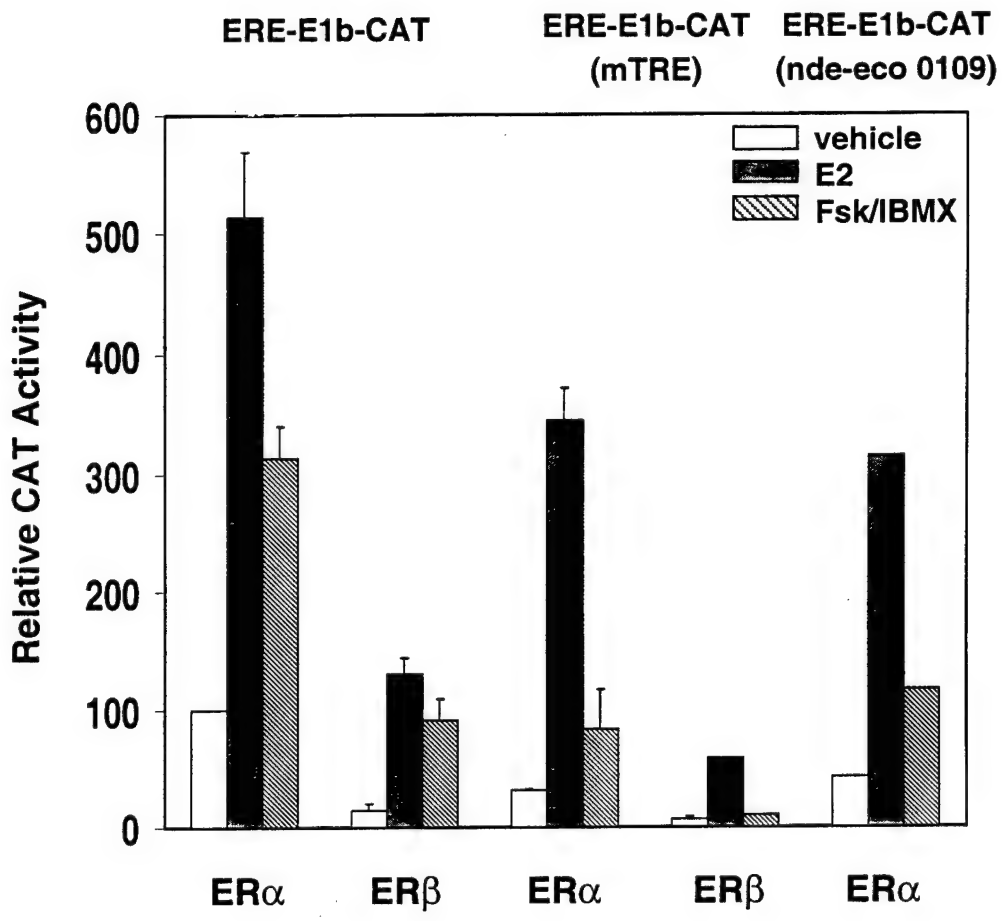

Figure 6: Putative AP-1 Response Element Contributes to PKA stimulation of ERα- and ERβ-dependent Gene Expression. HeLa cells were cotransfected with either 1 μg ERE-E1b-CAT or ERE-E1b-CAT (mTRE) reporter plasmid and 10 ng $pCMV_5$-hERα (ERα) or $pCXN_2$-hERβ (ERβ). Cells were stimulated with 1nM E2 or 10μM forskolin + 100 μM IBMX. CAT measurements were corrected for protein values and standardized to ERα activity on the ERE-E1b-CAT reporter in the presence of ethanol. Transfections were performed in duplicate and values represent the mean ± SEM of at least three experiments for the ERE-E1b-CAT and ERE-E1b-CAT(mTRE) and results from one experiment for ERE-E1b-CAT(NdeI-Eco0109).

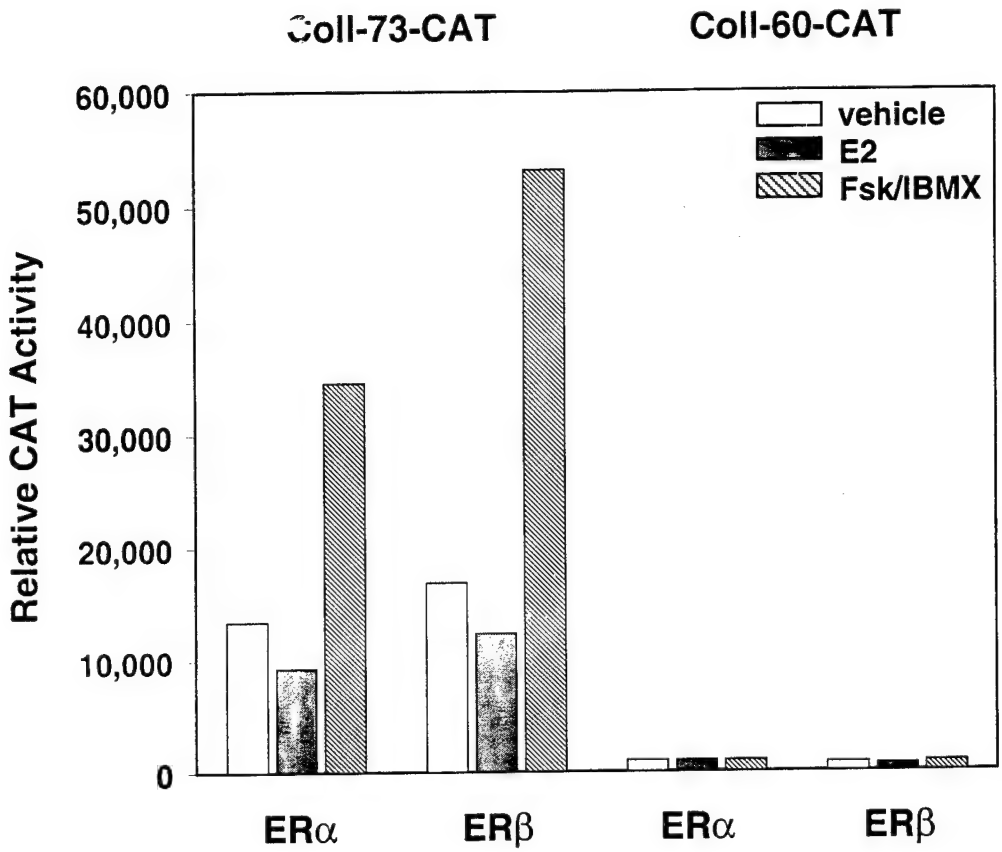

Figure 7: Forskolin and IBMX Stimulate AP-1 Activity. HeLa cells were cotransfected with either 1 μg Coll-73-CAT or Coll-60-CAT reporter plasmid along with 10 ng pCR3.1-hERα (ERα) or $pCXN_2$-hERβ (ERβ). Cells were treated with ethanol, 1nM E2, or 10μM forskolin + 100 μM IBMX and CAT measurements corrected for protein values. Transfections were performed in duplicate and values represent an individual experiment.

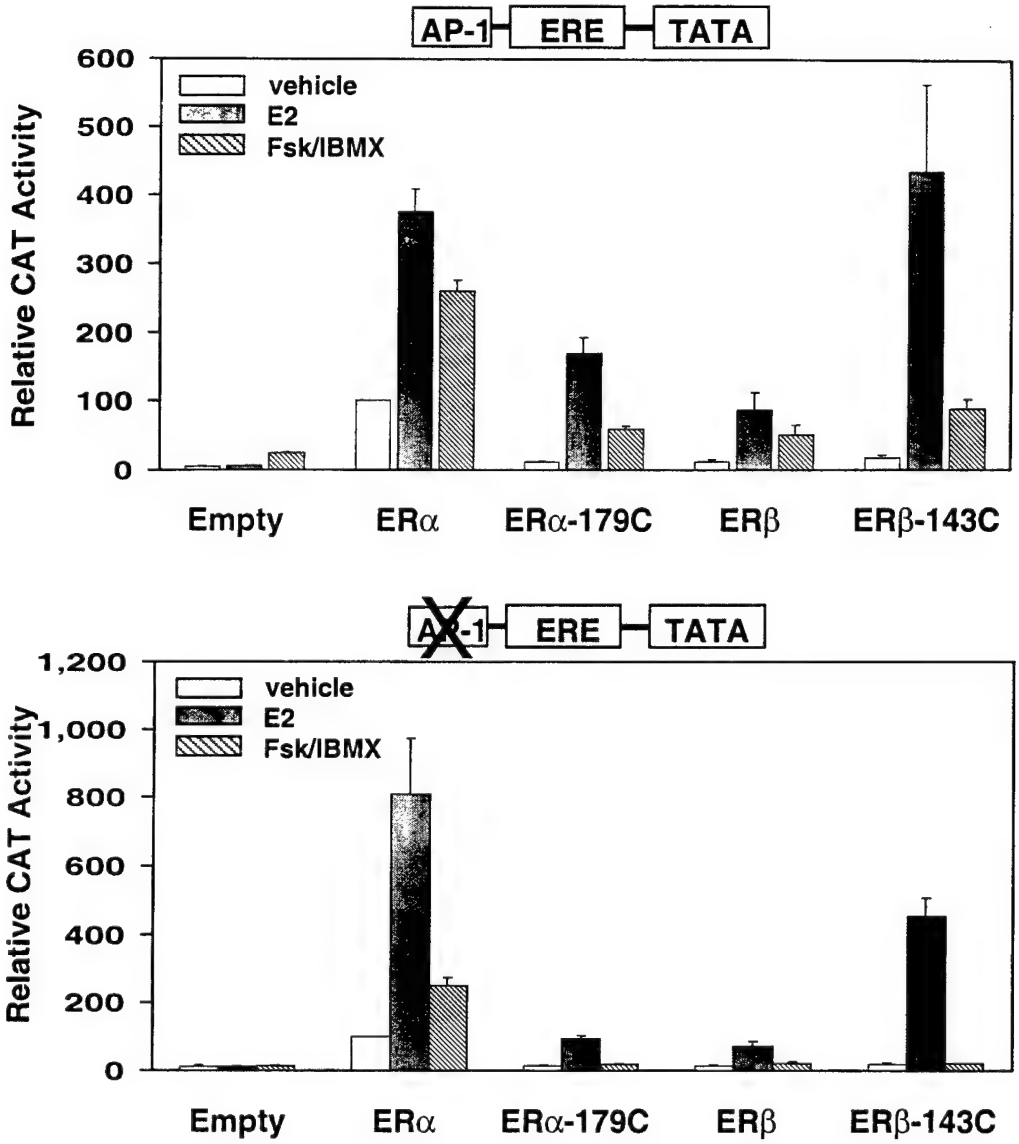

Figure 8: Amino Terminal A/B domains of ERα and ERβ are not Required for Functional Interaction with AP-1 Transcription Factors. HeLa cells were cotransfected with (**A**) 1 μg ERE-E1b-CAT or (**B**) 1 μg ERE-E1b-CAT (mTRE) reporter plasmid and either 10 ng pCR3.1, pCR3.1-hERα (ERα), pCXN$_2$-hERβ (ERβ), pCR3.1-hERα-179C (ERα-179C), or pCR3.1-hERβ-143C (ERβ-143C). Cells were stimulated with 1nM E2 or 10μM forskolin + 100 μM IBMX. CAT measurements were corrected for protein values and standardized to ERα activity on the ERE-E1b-CAT reporter in the presence of ethanol. Transfections were performed in duplicate and values represent the mean ± SEM of at least three experiments.

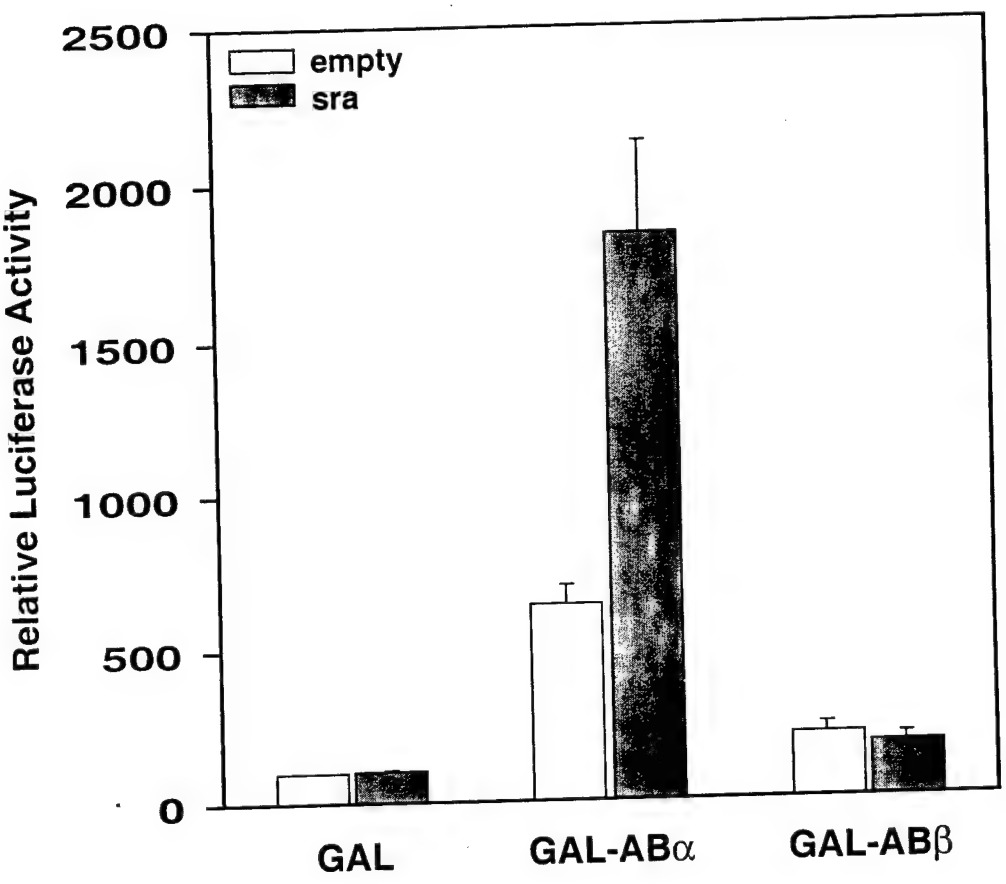

Figure 9: SRA Coactivates Amino Terminal A/B Domain of ERα but not ERβ. HeLa cells were cotransfected with 1 μg pG5-Luc reporter plasmid and either 1 μg pSCT-SRA (sra) or pSCT parent vector (empty) along with 100 ng pBind (GAL), pBind-ERα-A/B (GAL-ABα), or pBind-ERβ-A/B (GAL-ABβ). Luciferase measurements were corrected for protein values and standardized to ABα activity in the absence of transfected coactivator. Transfections were performed in duplicate and values represent the mean ± SEM of at least three experiments.

Stimulation of Estrogen Receptor α and β Transcriptional Activity by the RNA Coactivator, SRA.

Kevin M. Coleman, Vinh D. Lam, Rainer B. Lanz, Bert W. O'Malley, and Carolyn L. Smith. Dept. of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030

The biological effects of estrogens are mediated via the estrogen receptors α and β (ERα and ERβ). Recently, a novel coactivator which functions as an RNA transcript, termed Steroid receptor RNA Activator (SRA), was identified and shown to increase the ability of type I nuclear receptors to activate target gene expression, presumably through their activation function-1 (AF-1) domains. Because the transcriptional activity of the ERβ AF-1 has been shown to be very weak, we compared the ability of SRA to coactivate ERα and ERβ in transiently transfected HeLa cells. Transactivation assays demonstrated that exogenous SRA coactivated both ERα and ERβ by ~3-fold. Expression vectors encoding the A/B domain of each ER isotype fused to the Gal4 DNA binding domain were employed to study the effect of SRA on AF-1 transcriptional activity independent of other receptor domains. The ERα A/B domain activity was increased ~3-fold by SRA whereas the activity of the ERβ A/B domain was unchanged, indicating that the amino terminus of ERα, but not that of ERβ, can functionally interact with SRA. Successive deletions in the ERα amino terminus, which were used to map regions of the ERα A/B domain required for functional interaction with SRA, reduced overall receptor activity but did not substantially decrease the ability of SRA to coactivate ERα transcriptional activity. Moreover, ERα and ERβ deletion mutants lacking the entire A/B domain were coactivated by SRA, indicating that the amino termini are not required for SRA coactivation and that ER deletion mutants containing only activation function-2 (AF-2) can be stimulated by SRA. Previously, SRA was shown to exist in a complex with Steroid Receptor Coactivator-1 (SRC-1), primarily an AF-2 coactivator. We therefore assessed the combined effects of overexpression of SRA and SRC-1 on ER-dependent gene expression. Together, SRA and SRC-1 increased ERα and ERβ transcriptional activity to a greater extent than either coactivator alone, suggesting that these two molecules do not fulfill the same role in amplifying ER transcriptional responses. These data indicate that SRA, potentially as a complex with SRC-1, positively modulates the activity of the AF-1 and AF-2 domains of ERα but does not coactivate the AF-1 domain of ERβ. Thus, the lack of SRA effect on the A/B domain of the latter receptor may contribute to its relatively low activity in comparison to the corresponding domain of ERα. This work is supported by a grant from the Department of Defense to CLS (DAMD 17-98-1-8282).

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SRA Coactivates the AF-1 Domain of ERα but not ERβ, and Stimulates the Agonist Activity of the Partial Antiestrogen, 4-Hydroxytamoxifen

Kevin M. Coleman, Vinh Lam, Basem Jaber, Rainer B. Lanz AND Carolyn L. Smith*

Department of Molecular and Cellular Biology
Baylor College of Medicine
Houston, TX 77030-3498

Running title: Selective Coactivation of the A/B Domain of ERα

***Address correspondence to:** Carolyn L. Smith, Ph.D.
Department of Molecular and Cellular Biology
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
Tel: (713) 798-6235
Fax: (713) 790-1275
Email: carolyns@bcm.tmc.edu

ABSTRACT

Estrogenic responses are mediated by two estrogen receptors (ERs), ERα and ERβ, which possess poor sequence homology in their amino terminal (A/B) domains. This region encompasses the hormone-independent activation function-1 (AF-1), and functional differences in this domain are thought to contribute to the relatively weak transcriptional activity of ERβ in comparison to ERα, presumably through differences in coactivator interaction. Steroid receptor RNA activator (SRA) was identified as an AF-1-specific coactivator, and we therefore examined its ability to contribute to differences in ERα and ERβ transcriptional activity. In transient transfections, exogenous SRA expression increased ERα- and ERβ-dependent gene expression. However, when the A/B regions of ERα and ERβ were examined as GAL4 DNA binding domain fusions, SRA enhanced the transcriptional activity of GAL-ABα but not GAL-ABβ. In cells, SRA and SRC-1 are found in a steroid receptor coregulator complex, but cotransfection of expression plasmids for these coactivators yielded GAL-ABβ activity comparable to that obtained by SRC-1 alone. In contrast, SRA and SRC-1 synergistically coactivated the ERα A/B domain. The mixed antiestrogen, 4-hydroxytamoxifen (4HT) is an ERα AF-1 agonist. While SRA enhanced 4HT-stimulated ERα activity it was unable to convert 4HT to an ERβ agonist. However, deletion of the A/B region of both receptors demonstrated that AF-1 is not required for SRA-mediated coactivation and that SRA effects can be mediated by the carboxy-terminus of either ER. Thus, SRA is an ERα AF-1-specific coactivator that may account, at least in part, for differences in AF-1 transcriptional activity between ERα and ERβ, as well as the ability of tamoxifen to exert partial agonist activity on ERα.

INTRODUCTION

Estrogen receptors (ERs) are members of a superfamily of ligand-regulated transcription factors that stimulate gene expression in response to 17β-estradiol and other estrogenic compounds. There are two nuclear receptors for estrogens, ERα and ERβ, that are encoded by separate genes [[4266,5210]]. Sequence analysis and molecular studies reveal that these receptors can be divided into structural/functional regions, designated A through F (see Figure 1 [[469,1073]]). While the vast majority of work has been performed with ERα, it is clear that both receptors encompass a centrally located DNA binding domain, and two activation functions; the one located in the amino-terminal domain is referred to as activation function-1 (AF-1), while a second located in the carboxy-terminal ligand binding domain is named AF-2. The AF-1 domain is constitutively active, but functions in a cell- and promoter-dependent manner [[15,36]]. In contrast, the AF-2 domain contributes to the transcriptional activity of both ERα and ERβ in a ligand-dependent fashion. In many cellular contexts, both AF-1 and AF-2 of ERα are required for full receptor activity with the two activation domains exerting their effects on gene expression in a synergistic manner [[15,4000,363]]. In contrast, the AF-1 of ERβ appears to repress the activity of its corresponding AF-2 domain [[5366]].

The ability of nuclear receptors, including the estrogen receptors, to activate target gene expression is intimately linked to their ability to interact with coactivators. These proteins serve as bridging factors between estrogen receptors and components of the general transcriptional machinery, thus facilitating interactions between site-specific and general transcription factors [[5227]]. Several estrogen receptor coactivators have been identified and characterized [[5369]], including members of the p160 steroid receptor coactivator-1 (SRC-1) family (*e.g* SRC-1, TIF2/GRIP1 and RAC3). These proteins, in addition to their intrinsic activation domains also

encompass and/or interact with other proteins that possess histone acetyltransferase activity [[5044,5138,3989,5285]]. Thus, estrogen receptors recruit coactivator proteins with the ability to modify the local chromatin structure of target gene promoters and in so doing enhance gene expression [[5358]]. In addition, another ER coactivator, E6-AP, possesses ubiquitin ligase activity [[5061,5371]]. Although it is not clear what role if any, this enzymatic activity may play in regulating gene expression, it is becoming increasingly apparent that coactivators fulfill multiple functional roles in stimulating receptor-dependent gene expression. Interaction of protein coactivators with the ligand binding domain of estrogen receptors has been extensively studied and depends on the presence of agonists; antagonists do not promote coactivator binding to the ER [[5038,3993,3979]]. Crystallographic analyses indicate that an agonist-dependent shift in the orientation of helix 12 of the ERα ligand binding domain relative to the rest of this region facilitates binding of a GRIP1 coactivator peptide to the receptor [[5075]]. Furthermore, ligand-dependent alterations in the position of helix 12 have been noted for with the ligand binding domain of ERβ suggesting that these two receptor subtypes may interact with coactivators via common structural motifs [[5364]].

In an effort to identify and characterize potential AF-1 coactivator(s), interest has turned to the identification of molecules that interact with the amino-termini of nuclear receptors. Unlike the AF-2 domain, this region is poorly conserved in type I receptors (*e.g.* receptors for progestins, glucocorticoids, estrogens and androgens), and is absent in the type II receptors for thyroid hormone, retinoic acid and vitamin D. Recently, by a yeast two-hybrid approach that employed the A/B domain of progesterone receptor-A (PR_A) as bait, a novel coactivator was identified that stimulated the transcriptional activity of type I, but not type II receptors [[5060]]. Interestingly, this molecule enhanced PR_B transcriptional activity in the presence of

cycloheximide, regardless of the presence of termination codons in the RNA. It was therefore suggested to act as an RNA molecule [[5060]] and was termed steroid receptor RNA activator (SRA). In T47D cells, this molecule is found in a large multiprotein, ribonucleoprotein complex that also contains SRC-1, and it appears to activate PR_B- and glucocorticoid receptor (GR)-dependent transcription via their AF-1 domains [[5060]].

Differences in the ability of ERα and ERβ to activate estrogen-dependent target gene expression in transient transfection assays have been attributed to functional inequalities of their poorly conserved A/B domains, presumably due to distinct AF-1 activity [[5366,5264,5263]]. Tamoxifen stimulation of classical ER-mediated gene expression is dependent on the receptor's AF-1 domain, and it has been demonstrated that this antiestrogen blocks AF-2, but not AF-1, function [[3939,4000,3266,16]]. The ability of tamoxifen to act as a partial agonist of ERα, but not ERβ on ERE-containing target genes is therefore also likely a reflection of the distinct activities of the ERα and ERβ AF-1 domains [[5366,5264,5370,5024]]. Several protein coactivators (*e.g.* SRC-1 family members) interact with the A/B domains of both ERα and ERβ and therefore are not likely to be responsible for the differences in the activities of these two ER subtypes [[5023,5033,5120]]. However, a p68 RNA helicase with coactivator activity was recently shown to act in an ERα-specific manner [[5363]], suggesting that functional differences between ERα and ERβ may result from distinct receptor-coactivator interactions. In order to determine whether SRA functional interactions with ERα and ERβ were comparable, we have examined the ability of SRA to stimulate the transcriptional activity of each receptor subtype in the presence of ER agonists and antagonists, as well as their isolated A/B domains. In addition, we examined the ability of SRA and SRC-1 to functionally interact with respect to stimulating

ERα and ERβ transcriptional activity. Our results indicate that SRA acts as a coactivator of the A/B domain of ERα but not ERβ, and that SRA and SRC-1 can cooperate with one another in certain contexts to activate transcription, suggesting that SRA can enhance AF-1 and/or AF-2 function in a receptor-dependent manner. Thus, SRA is a selective ERα AF-1 coactivator, and this may explain, in part, the differences in transcriptional activity observed for ERα and ERβ.

MATERIALS AND METHODS

Chemicals

17β-Estradiol (E_2) was obtained from Sigma Chemical Company (St. Louis, MO). The antiestrogens, ICI 182,780 and 4HT were gifts from Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK) and D. Salin-Drouin (Laboratoires Besins Iscovesco, Paris, France), respectively.

Plasmid DNAs

The mammalian expression plasmids for human ERα [pCMV$_5$-hERα [[2208]]; pCR3.1-hERα, ref. [[5061]]; or pRST$_7$-hERα, ref. [[4000]]], the full-length [pCXN$_2$-hERβ, ref [[5216]]] and a short form of human ERβ composed of amino acids 46-530 (pCMV$_5$-hERβ, ref. [[5210]]), and the AF-2 mutants of hERα (pRST$_7$-hER-3x and pRST$_7$-hER-TAF2-3x, ref. [[4000]]) were described previously as were the synthetic target genes, pERE-E1b-Luc [[5061]] and pC3-Luc [[4000]]. The pG5-Luc target gene, which contains five binding sites for the GAL4 DNA binding domain (DBD) fused to the luciferase gene, was obtained from Promega Corporation. The mammalian expression vector for SRA (pSCT-SRA) and its corresponding parent vector (pSCT) have been used in a previous study [[5060]].

The mammalian expression vector for SRC-1e (pCR3.1-SRC-1e) was constructed as follows: Oligo(dT)$_{12-18}$ primers (Life Technologies) were used to reverse transcribe total RNA isolated from HeLa cells and the resulting material was PCR amplified with 5′ (5′-TGTGTTCAGTCAAGCTGTCC-3′) and 3′ (5′-GATGTCTGATCACCTTACGAG-3′) primers (the latter containing an *Spe*I site, underlined) to produce a 327 base pair (bp) portion of the SRC-1e cDNA. This fragment was digested with *BstZ17*I and *Spe*I and substituted for a 556 bp *BstZ17*I-*Xba*I region of the expression plasmid for SRC-1a (pCR3.1-SRC 1a, ref. [[5065]]) to yield pCR3.1-SRC-1e. In order to make the pCMV$_5$-hERα-87C expression plasmid, PCR was employed to amplify the portion of pCMV$_5$-hERα encoding amino acids 87-315 using the 5′ primer 5′-GCCCGCGGACC**ATG**GCGGCGTTCGGCTCCAACGGC-3′ [containing a *Sst*II (underlined) and start codon (bold) followed by the codons for amino acids 87-93] and the 3′ primer 5′-TCCCTGACGGCCGACCAGATG-3′ containing an *Xma*III site (underlined). This PCR product (698 bp) was cloned into the pCR3.1 expression vector using the TA cloning kit (Invitrogen). The *Sst*II-*Xma*III fragment from this vector was excised, and substituted for a 974 bp *Sst*II-*Xma*III fragment of wild type pCMV$_5$-hERα. An expression vector encoding an ERα mutant lacking the first 108 amino acids (pCR3.1-hERα-109C) was constructed by digesting the wild type ERα expression vector (pCR3.1-hERα) with *Sac*II to remove the first 324 nucleotides of the coding region and religated. The constructs for hERα-179C (pCR3.1-hERα-179C) and hERβ-143C (pCR3.1-hERβ-143C) were made by PCR using the primers 5′-ACCATGGCCAAGGAGACTCGCTACTGT-3′ and 5′-CTCTCAGACTGTGGCAGGGAAACC-3′ to amplify the segment of pCMV$_5$-hERα encoding amino acids 179 to 595 and the primers 5′-ACCATGAAGAGGGATGCTCACTTCTGC-3′ and

5′-GCGTCACTGAGACTGTGGGTTCTG -3′ to PCR amplify the segment of $pCXN_2$-hERβ cDNA encoding residues 143 to 530, respectively. Each of the resulting PCR fragments was cloned into the pCR3.1 expression plasmid using the TA cloning kit. All PCR products were verified by sequence analysis to ensure that errors did not occur during their synthesis.

The mammalian expression plasmids for the A/B domain of ERα and ERβ fused to the GAL4 DNA binding domain were constructed as follows. First, PCR amplification was performed using the primers 5′-TAAAACGGGCGGGATCCCGATGACCATGACCCTCCACACCAAA-3′ and 5′-CCCCGGGATCCTTAAGTCTCCTTGGCAGATTCCATAGC-3′ to amplify a portion of the human ERα cDNA ($pCMV_5$-hERα) encoding amino acids 1-182, while the primers 5′-GGGCCGGGATCCCGATGGATATAAAAAACTCACCATCT-3′ and 5′-CCCCGGGATCCTTAAGCATCCCTCTTTGAACCTGG-3′ were used to amplify the region of the human ERβ ($pCXN_2$-hERβ) cDNA encoding amino acids 1-146. Each of these primers possesses a *Bam*H1 restriction site (underlined). The resulting cDNA fragments were subcloned into the pCR3.1 TA cloning vector to produce pCR3.1-hER-ABα and pCR3.1-hER-ABβ, respectively. Plasmids were digested with *Bam*H1 and each of the resulting cDNA fragments was subcloned into the *Bam*H1 restriction site of the pBind vector (Promega), such that the coding sequences for each ER A/B domain is in-frame with and downstream of the coding sequence for amino acids 1-147 of the GAL4 DNA binding domain. The resulting vectors were sequenced to ensure that no errors were introduced during plasmid construction.

Cell Culture and Transfections

HeLa (human cervical carcinoma) cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty four hours prior to transfections, cells were plated in six-well culture dishes at a density of 3×10^5 cells per well in phenol red-free DMEM with 5% charcoal-stripped fetal bovine serum (sFBS). DNA was introduced into cells in the indicated amounts using Lipofectin (Life Technologies), according to the manufacturer's guidelines. Eight hours later, serum-free media was replaced with phenol red-free DMEM supplemented with 5% sFBS. Twelve hours thereafter, cells were treated with the indicated amounts of various hormones. After 7 hours of hormone treatment, cells were harvested and extracts were assayed for luciferase activity using the Luciferase Assay Systems kit (Promega) and a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). Relative luciferase units were normalized to total cellular protein, as determined by Bio-Rad Protein Assay. Experiments were done in duplicate and values represent the mean ± SEM of at least three individual experiments.

HepG2 (hepatocellular carcinoma) cells were maintained in similar growth conditions as those for HeLa cells. Twenty four hours prior to transfection, cells were plated at a density of $8x10^5$ cells per well in six-well culture plates in phenol red-free DMEM with 5% sFBS. DNA was introduced into cells using Lipofectamine (Life Technologies), according to the manufacturer's recommendation. Following 8 hours of transfection, media was changed to phenol red-free DMEM with 5% sFBS. Twenty hours later cells were treated with the indicated amounts of hormone for 20-24 hours. Cells were harvested and cell lysates prepared and assayed for luciferase activity as described above. Experiments were done in triplicate and values are reported as the mean ± SEM from at least three individual experiments.

RESULTS

It has been previously demonstrated that the RNA coactivator, SRA, stimulates the transcriptional activity of several type I nuclear receptors including the PR_B, GR, androgen receptor (AR) and Eκα, and experiments with deletion mutants of both PR_B and GR indicated that their AF-1 domains were required for SRA to enhance transcriptional activity [[5060]]. The two subtypes of estrogen receptors, ERα and ERβ, are poorly conserved in their A/B domains which share ~26% identity, while the DNA binding and ligand binding domains (84% and 58%, respectively) are relatively well conserved. In order to assess whether SRA could coactivate ERα and ERβ transcriptional activity in a similar fashion, SRA was overexpressed in cells transfected with expression vectors for the full-length form of human ERα or ERβ (**Figure 1**) and the estrogen receptor-dependent synthetic target gene, ERE-E1b-Luc, which consists of a consensus estrogen response element linked upstream of a TATA box and luciferase reporter gene. When cells were treated with 1 nM estradiol, both ERα and ERβ activated luciferase expression (**Figure 2**), and as expected, full-length ERβ was ~50% less active in comparison to ERα (data not shown). However, the ability of SRA overexpression to enhance receptor-dependent target gene expression did not vary; both ERα and ERβ activities were stimulated approximately 3-fold. Therefore, SRA is a coactivator for ERα and ERβ which is consistent with SRA being selective for type I nuclear receptors.

The ability of the mixed antiestrogen, 4-hydroxytamoxifen (4HT) to stimulate ERα transcriptional activity is dependent upon the amino-terminal AF-1 function, and the cell and promoter context in which gene activation studies are performed [[16,4000]]. Furthermore,

overexpression of protein coactivators including SRC-1 and GRIP1 stimulates the transcriptional activity of ERα liganded with the mixed antiestrogen, 4HT [[5023,4225,5363]]. Therefore, because SRA is an AF-1-dependent coactivator of PR_B and GR, its ability to enhance 4HT-stimulated transcriptional activity of ERα and ERβ was assessed (**Figure 3**). As anticipated, in the absence of exogenous SRA, 4HT is a relatively poor agonist in HeLa cells. However, overexpression of SRA increased the ability of 4HT to activate gene expression in comparison to estradiol (~4-fold *versus* 3-fold, respectively), suggesting that ERα liganded with this partial agonist/antagonist was able to functionally interact with SRA. In contrast, other investigators have been unable to demonstrate activation of ERβ-dependent transcription by 4HT even though ERβ binds to 4HT with an affinity comparable to that of ERα [[4263,5024,4266,5255]]. In order to determine if SRA overexpression might partially overcome ERβ resistance to 4HT agonist activity, luciferase gene expression was measured in HeLa cells transfected with expression plasmids for ERβ and SRA, and treated with 4HT. As shown in figure 3, 4HT alone was unable to stimulate the transcriptional activity of ERβ in this system and SRA overexpression was unable to rescue the inability of 4HT to stimulate ERβ-dependent gene expression.

In order to determine if SRA overexpression could alter the transactivation induced by a second class of antiestrogens, the ability of the pure antiestrogen ICI 182,780 to activate transcription in the absence and presence of exogenous SRA was also assessed (**Figure 3**). This antiestrogen inhibits ERα and ERβ transcriptional activity in most contexts in which it has been tested, and in HeLa cells it is unable to stimulate the activity of either ERα or ERβ whether or not SRA was cotransfected. These data indicate that the ability of SRA to stimulate the transcriptional activity of ERs liganded with antiestrogens depends on the nature of the

antiestrogen [partial (4HT) *versus* pure (ICI 182,780)] and the subtype of estrogen receptor (α *versus* β).

The observation that SRA overexpression resulted in enhanced transcription stimulated by 4HT was confirmed and extended in HepG2 cells using the pC3-Luc target gene. The mixed antiestrogen, 4HT, is a relatively good agonist in Hep G2 cells [[4000]], and ERα activity is highly dependent upon AF-1 activity, whereas in HeLa cells both AF-1 and AF-2 are usually required for robust transcriptional activity [[16]]. The pC3-Luc target gene consists of the -1807 to +58 region of the human complement-3 (C3) gene fused to a luciferase reporter and contains three imperfect estrogen response elements [[3987]]. As expected [[4225]], 100 nM 4HT was able to stimulate the transcriptional activity of ERα in HepG2 cells as well as 10 nM estradiol (**Figure 4**). Furthermore, SRA overexpression stimulated the transcriptional activity of ERα in the presence of 4HT to the same extent as when the receptor was liganded by 17β-estradiol. When the experiment was performed with an expression vector for ERβ, the luciferase activity induced by E2 was only ~ 40% as great as that stimulated by ERα (data not shown), and consistent with its refractoriness to mixed antiestrogens, no activation of target gene expression was detected in 4HT-treated cells. Exogenous SRA, introduced via transient transfection, resulted in coactivation of luciferase gene expression only when cells were treated with estrogen; 4HT-liganded ERβ was still unable to stimulate target gene activity. Consistent with the results obtained in HeLa cells, the pure antiestrogen ICI 182,780 was unable to activate transcriptional activity of either ERα or ERβ, and SRA overexpression had no effect on the ability of these receptor-ligand complexes to activate transcription.

Taken together, the above data indicated that SRA can coactivate estrogen-stimulated ERα- and ERβ-dependent gene expression even though the amino-termini of these receptors share little identity. However, differences between the two receptors are observed when cells are treated with 4HT in the presence or absence of exogenous SRA, suggesting that their respective AF-1 domains are not functionally homologous. Therefore, in order to determine if SRA could coactivate the transcriptional activity of the AF-1 domains of each receptor subtype isolated from the context of the remainder of the receptor molecule, expression vectors encoding fusions of the GAL4 DBD to either the A/B domain of ERα (GAL-ABα) or ERβ (GAL-ABβ) were made. These were cotransfected into HeLa cells with a target gene containing five GAL4 DNA binding sites (pG5-Luc) in the presence or absence of an expression vector for SRA. As shown in **Figure 5A**, the A/Bα domain in the absence of exogenous coactivator has considerable transcriptional activity in comparison to the GAL4 DBD alone. However, the transcriptional activity of GAL-ABβ is very weak in comparison to the GAL-ABα chimeric protein, indicating that the two A/B regions are distinct in their ability to activate target gene expression, and that the AF-1 activity of ERα is significantly stronger than that of ERβ in this cellular context. The ability of SRA to coactivate these regions independently of AF-2 was tested in parallel by SRA overexpression in transient transfection assays. As expected, SRA had no effect on the minimal activity seen in cells transfected with the GAL4 DBD alone. In contrast, exogenous SRA stimulated the ability of the GAL-ABα chimera to activate target gene expression nearly 3-fold, but did not enhance the transcriptional activity of the GAL-ABβ chimera. This suggests that SRA is an ERα-specific coactivator of the AF-1, and that SRA must coactivate ERβ through a region other than the A/B domain.

To confirm that ERα could be activated via its AF-1 domain in the context of a full length receptor, the ability of a mutant ERα (ERα-3x) in which three amino acids in the core AF-2 domain are mutated (D538A/E542A/D545A), was assessed for its ability to be coactivated by exogenous SRA. These mutations severely inhibit ERα interaction with the nuclear receptor interaction domains of SRC-1 and GRIP1 [[5380]]. As shown in **Figure 5B**, the gene expression stimulated by this receptor was increased by SRA, although to a lesser extent than wild type receptor (2.6-fold *versus* 3.4-fold, respectively). However, when the AF-1 domain was deleted in addition to the AF-2 point mutations to yield the ERα-TAF2-3x mutant, no hormone-induced transcriptional activity was observed and SRA overexpression was unable to substantially increase luciferase gene expression. As a further control, the effect of exogenous SRA in cells transfected with the target gene and the parent expression vector lacking any ERα cDNA was assessed, and no change in luciferase activity was noted. Taken together, these data demonstrate that in the context of the holoreceptor an intact AF-2 domain is not required for SRA to coactivate ERα transcriptional activity.

To date, it is unclear whether SRA binds directly to type I receptors or via an intermediary protein. It has been shown previously by coimmunoprecipitation experiments that SRA and SRC-1 can physically associate with one another in *Xenopus* oocytes and that SRC-1 could facilitate detection of SRA in a complex containing an AF-1 deletion mutant of the androgen receptor [[5060]]. Thus, it is possible that SRA may interact with steroid receptors via SRC-1. Although SRC-1 was identified as an AF-2-binding coactivator protein [[3096]], evidence has been obtained demonstrating that it also can interact with the amino-terminus of ERα and ERβ [[5023,5033,5120]]. Therefore, expression vectors for SRC-1e, the SRC isoform with greatest ER enhancing activity [[5038]], and SRA were transfected into HeLa cells to determine if

coexpression of these coactivators would facilitate coactivation of the ERα or ERβ AF-1 activity in a cooperative manner. As shown in **Figure 6**, coexpression of SRA and SRC-1 increased the transcriptional activity of the ERα A/B domain to an extent greater than either coactivator alone indicating that these two proteins may be part of a functionally interactive pathway able to modulate ERα AF-1 activity. In contrast, only SRC-1 was able to stimulate the transcriptional activity of the ERβ A/B domain, and the addition of exogenous SRA did not modulate the magnitude of this response. This finding is consistent with the inability of SRA alone to stimulate the transcriptional activity of the GAL-ABβ chimera, and suggests that SRA functional and/or physical interactions with A/B domains of type I receptors are not simply the results of SRA interactions with SRC-1. Thus, these data further emphasize that SRA has the potential to act via the AF-1 domain of ERα but not ERβ, and that the molecular nature of this cooperative response requires appropriate interactions of SRA and SRC-1 within the context of the A/B domain, and not simply interaction of SRC-1 and subsequent recruitment of SRA.

The results obtained above suggested that SRA coactivates ERα via its AF-1 domain, but that this region of ERβ was insufficient for a functional interaction with this RNA coactivator. Therefore, in order to determine whether the AF-1 domain of ERα was required for SRA coactivation, and whether SRA could coactivate ERβ via its ligand binding domain, a series of A/B deletion mutants of ERα and ERβ were tested for their ability to activate target gene expression in the absence and presence of exogenous SRA. Expression vectors for the truncated forms of ERα were cotransfected into HeLa cells along with the ERE-E1b-Luc synthetic target gene and the transcriptional activity in the presence of vehicle or 1 nM E2 was assessed. Deletion of the first 86 amino acids of ERα reduced its transcriptional activity, in the presence of

estradiol, to ~60% of wild type receptor activity (**Figure 7A**). Deletion of amino acids 1-108 also reduced activity to approximately the same extent (**Figure 7B**) which is consistent with the concept that these amino acids contribute to ERα AF-1 activity stimulated by estradiol [[5256]]. However, this region was not required for SRA coactivation, since deletion of these residues did not block the enhancement of the transcriptional activity of the resulting receptor mutants. In order to determine if any portion of the A/B domain was required for this receptor to be coactivated by SRA, a more extensive deletion mutant of ERα lacking the entire A/B domain (ERα-179C) was generated and examined for its ability to support SRA coactivation of target gene expression (**Figure 7B**). Surprisingly, the coactivator retained its ability to enhance luciferase activity indicating that while SRA could activate transcription via the A/B domain, this region was not necessary for SRA coactivation of ERα-dependent target gene expression.

The first human ERβ cDNAs cloned did not encode the full-length receptor [[5210,4268]], and the ability of a truncated form of ERβ, lacking the first 45 amino acids also was tested for its ability to be coactivated by exogenous SRA expression. As shown in **Figure 7C**, the transcriptional activity of this short form of ERβ was stimulated as well as wild type receptor by SRA, suggesting that the extreme amino-terminus of ERβ is not required for coactivation by SRA. The ability of an ERβ mutant lacking all A/B domain sequences (ERβ-143C) was next examined and as shown in **Figure 7D**, SRA was able to stimulate the transcriptional activity of this mutant receptor to an extent comparable to wild type ERβ, suggesting that coactivation of ERβ is mediated by the remaining carboxy-terminal portion of the receptor. Taken together, these data suggest that SRA, while an ERα-specific coactivator of the AF-1 domain, also

possesses the ability to coactivate transcription via the ligand and/or DNA binding domains of both estrogen receptor subtypes.

Since the carboxy-terminal portion of both ER subtypes was able to be coactivated by SRA and SRC-1, the ability of these two coactivators to functionally interact with ERα and ERβ was assessed in the context of the respective full length receptors. As shown in **Figure 8A**, under conditions where SRA and SRC-1 individually are weak activators of ERα-dependent target gene expression, transient transfection of the expression plasmids for the RNA and protein coactivators resulted in gene expression, as measured by luciferase activity, greater than either coactivator alone. When similar experiments were performed with ERβ, coexpression of SRA and SRC-1 resulted in luciferase activity that also was greater than additive (**Figure 8B**). Taken together, this suggests that SRA and SRC-1 can functionally interact in the context of the full-length receptor, and that the carboxy-terminal portion of ERβ, but not ERα, is required to support this transcriptional response.

DISCUSSION

The identification of a second receptor for estrogens, ERβ, has substantially increased the potential complexity of estrogen action *in vivo*, and much effort is currently underway to resolve both mechanistic and physiological consequences of this second estrogen receptor subtype. Although both receptors bind estradiol with nearly equivalent affinity [[5255,4263]], their abilities to stimulate target gene transcription are distinct. In general, ERα is a superior activator with respect to the magnitude of ERE-containing target gene expression in transient transfections [[5263,4268,5264,5265,5370]], although it is possible that target genes remain to be identified

where ERβ will be a more robust activator of transcription. Alternatively, ERβ may be considered as a negative regulator of estrogen action since it inhibits ERα transcriptional activity in some contexts, presumably through a heterodimerization mechanism [[5366,5265]]. In view of the divergent amino-terminal sequences of ERα and ERβ, and the reported ability of SRA to activate the AF-1 domain of steroid receptors [[5216,5367,5060]], as well as to better understand the mechanistic basis for differences in the ability of ERα and ERβ to stimulate gene expression, we have conducted a series of experiments to characterize the ability of these receptors to functionally interact with the novel RNA coactivator, SRA.

Exogenous SRA increased levels of both ERα- and ERβ-dependent gene expression suggesting that the relative differences in these two receptor subtypes to activate target gene transcription was not due to an absolute inability of SRA to functionally interact with ERβ. Furthermore, the ability of SRA to enhance the transcriptional activity of either receptor was not restricted to a specific cell or promoter type since SRA coactivated ERα and ERβ transcriptional activity in HeLa and HepG2 cells on the ERE-E1b-Luc and pC3-Luc promoters, respectively. Thus, SRA coactivates ERα and ERβ transcriptional activity. In addition, SRA and SRC-1 appear to work together, since activation of target gene expression is greater following transfection of expression vectors for both coactivators than would be anticipated from either coactivator alone, and this is consistent with the presence of SRA in SRC-1, but not p300 containing complexes isolated from T47D cells [[5060]]. At present the mechanism(s) by which SRA alters ER-dependent target gene expression is unclear. In general, there is an increasing appreciation of the potential roles that RNA plays in regulating transcription, including even functions more generally associated with proteins. For example, RNA may regulate gene expression in *trans* by assuming a structural role and recruiting proteins to promoters [[5379]],

altering mRNA translation or stability by antisense RNA-RNA interactions [[5378,5376]], or by regulating the enzymatic activity of associated proteins that affect transcription factor activity [[5377]].

The ability of SRA to affect the transcriptional activity of A/B domains isolated from the remaining portion of their respective receptors was assessed to determine the ability of SRA to specifically serve as an AF-1 coactivator. We first characterized the activity of each A/B domain in the absence of exogenous coactivators. Interestingly, when tethered to the GAL4 DBD, the A/B domain of ERα was significantly more active than the corresponding region of ERβ. This work confirms and extends earlier observations in which it was shown that a GAL4 DBD fusion with 95 amino acids of the N-terminal region of ERβ (corresponding to amino acids 54 - 148 of the human ERβ full length sequence) was less active than a chimeric protein comprised of the full length A/B domain of ERα (amino acids 1-182) linked to the GAL4 DBD [[5366]], and indicates that even the full length ERβ A/B domain (amino acids 1-143) is less active than the corresponding region of ERα. Similar results have also been shown in HepG2 cells [[5263]]. This also supports the hypothesis that functional differences in ER subtypes arise, at least in part, from differences in the activity of the amino-terminal domains.

The basis for the differences in ERα and ERβ A/B domain activities are presumably related to the relative ability of these regions to physically and/or functionally interact with coactivators. It had been shown previously that this region of both receptors could interact with SRC-1, suggesting that the relative inactivity of the ERβ A/B domain is not due to a failure of this region to bind p160 coactivators [[5023,5120]]. Although it is not clear whether SRA binds directly to the A/B domain of nuclear receptors, there is a distinct difference in the ability of

SRA to functionally interact with this region of ERα and ERβ when they are isolated from their respective DNA and ligand binding domains, and this likely contributes to the relatively poor transcriptional activity of the ERβ A/B domain. This is even more striking because previous studies had demonstrated that SRA is found in a complex in cells with SRC-1 [[5060]], and we hypothesized that it might be possible for SRA to interact with the A/B domain of either receptor subtype via this p160 coactivator. However, a synergistic response was observed only when SRA and SRC-1 were co-expressed in the GAL-ABα, but not GAL-ABβ transfected cells, further emphasizing the inability of SRA to functionally interact with the A/B domain of ERβ. This result also argues that the functional interaction of SRA with the ERα AF-1 is not simply the result of its recruitment to that region via SRC-1, and suggests that SRA either interacts directly with the A/B domain of ERα but not ERβ, or that it interacts with and/or requires other factors that selectively associate with the A/B domain of ERα.

Recently, the identification of another AF-1 coactivator for ERα was reported [[5363]]. The p68 RNA helicase was isolated as an ERα AF-1 domain interacting protein that selectively enhanced the activity of the A/B domain of ERα, but not ERβ in COS-1 cells [[5363]]. Although it is tempting to speculate that p68 and SRA function together, p68 does not coactivate the AF-1 domain of ERα in HeLa cells [[5363]]. Furthermore, p68 RNA helicase does not activate the AF-1 domains of other receptors, such as the androgen receptor, that are targets for SRA effects [[5363,5060]]. In addition, the ATP-dependent RNA helicase activity of p68 can be abolished by point mutation and the resulting protein still retains it transcription enhancing activity [[5363]]. Taken together, the p68 RNA helicase and SRA appear to be functionally distinct, but their existence argues that there are AF-1-specific coactivators which act in a cell-

type- and/or receptor-specific manner, and are likely to contribute to the distinct transcriptional activity of ERβ in comparison to ERα.

The ability of SRA to coactivate the transcriptional activity of the ERβ holoreceptor, but not its AF-1 suggested that SRA could modulate receptor function through a region other than the A/B domain. Sequential deletion of the A/B domain of ERα demonstrated that the AF-1 region required for tamoxifen (amino acids 1-88) or estradiol (89-108) agonist activity [[5256]] was not required for SRA functional interactions. Furthermore, the ability of SRA to coactivate mutant forms of ERα and ERβ lacking their entire A/B domains (ERα-179C and ERβ-143C, respectively) also indicates that SRA, like the p160 SRC-1 family of coactivators [[5033,5023]] can stimulate transcriptional activity through the amino- and carboxy-terminal portions of the receptor. The ERα mutant in which the AF-1 deletion was combined with point mutations of AF-2 (ERα-179C-3x) further confirms that this coactivator modifies ER transcriptional activity through the receptor's activation domains. Although SRA interactions with the ligand binding domain of nuclear receptors are not defined, SRA could be detected in complexes immunoprecipitated with an androgen receptor antibody from *Xenopus* oocytes expressing an AF-1 deletion mutant of androgen receptor only when SRC-1 was coexpressed [[5060]]. Thus, SRA is able to physically interact with the carboxy-terminal portion of a nuclear receptor through an interaction with SRC-1, suggesting that SRA can enhance the transcriptional activity of the AF-2 domains of ERα and ERβ through a similar mechanism. This result also highlights the differences between the amino- and carboxy-terminal regions with respect to SRA coactivation, since coexpression of SRC-1 was insufficient to enable SRA coactivation of the ERβ AF-1 domain.

Tamoxifen is a member of a class of drugs referred to as selective estrogen receptor modulators (SERMs), that can manifest agonist or antagonist activity depending on the cell, promoter and estrogen receptor subtype [[5356]]. The crystal structure of the ERα ligand binding domain has been solved [[5075,5076,5045]], and the surface of the receptor to which p160 coactivators bind has been defined [[5372]]. In the presence of tamoxifen the LBD adopts a conformation that produces an intramolecular interaction of helix 12 (amino acids 536 to 544) with the receptor's coactivator interaction surface that occludes binding of a GRIP-derived coactivator peptide [[5075]]. Thus, the position of helix 12 induced by tamoxifen is likely to block coactivator interaction with the ligand binding domain, and in so doing antagonize AF-2 activity. Tamoxifen does not inhibit the AF-1 of ERα, and deletion experiments indicate that the A/B region is required for tamoxifen-stimulated gene expression [[16,3266,5256]]. Thus, this SERM's agonistic properties are believed to be dependent on cell-specific coactivator interactions with ERα [[5356]]. The inability of tamoxifen to stimulate ERβ transcriptional activity on ERE-containing target genes implies an AF-1 deficiency relative to ERα, and highlights the importance of defining AF-1-coactivator interactions [[5366,5264,5370,5024]]. Since tamoxifen-stimulated ERα activation is enhanced by SRC-1, GRIP1 and RAC3 [[4225,5023,5363]], and at least SRC-1 can interact with the A/B domain of ERβ [[5120]], the p160 coactivators are unlikely to be the primary basis for differences in ERα and ERβ transcriptional activity in the presence of tamoxifen. The identity of SRA as an ERα-specific AF-1 coactivator suggests that this positive coregulator may contribute to the tissue-specific activity of SERMs. Furthermore, this suggests that alterations in SRA expression may have a significant impact on a tissue's responsiveness to tamoxifen. Interestingly, elevated expression of an SRA variant has been strongly correlated with breast tumors of high grade [[5373]], raising

the intriguing possibility that changes in SRA expression may have implications for the sensitivity of breast tumors to tamoxifen therapy.

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REFERENCES

FIGURE LEGENDS

Figure 1: Schematic representation of wild type and mutant forms of ERα and ERβ used in this study. A schematic of the structural regions (A-F) and functional domains [DNA binding and activation functions (AF-1 and AF-2)] are shown on top. The A/B domains of ERα (fine diagonal) and ERβ (speckled), and the ligand binding domains of ERα (solid) and ERβ (coarse diagonal) are indicated below.

Figure 2: Coactivation of human ERα and ERβ by SRA overexpression. HeLa cells were cotransfected with 40 ng of the indicated ER expression vector ($pCMV_5$-hERα or $pCXN_2$-hERβ) and 1500 ng of ERE-E1b-Luc in the presence or absence of 1000 ng of SCT-SRA and treated with ethanol (NH) or 1 nM 17β-estradiol (E2). Values are the average ± SEM of three independent experiments standardized to the luciferase data obtained in the presence of estrogen and absence of transfected SRA expression vectors.

Figure 3: SRA enhances the agonist activity of 4-hydroxytamoxifen on ERα, but not ERβ. HeLa cells were cotransfected with 40 ng of the indicated ER expression vector ($pCMV_5$-hERα or $pCXN_2$-hERβ) and 1500 ng of ERE-E1b-Luc in the presence or absence of 1000 ng of SCT-SRA and treated with ethanol (NH), 1 nM 17β-estradiol (E2), 100 nM 4-hydroxytamoxifen (4HT) or 100 nM ICI 182,780 (ICI). Values are the average ± SEM of 4-7 independent experiments standardized to the luciferase data obtained in the presence of estrogen and absence of transfected SRA expression vectors.

Figure 4: SRA coactivates ERα transcriptional activity in the presence of mixed, but not pure antiestrogens in HepG2 cells. Cells were cotransfected with 50 ng of the indicated ER expression vector ($pCMV_5$-hERα or $pCXN_2$-hERβ) and 1000 ng of pC3-Luc in the presence or absence of 1000 ng of SCT-SRA and treated with ethanol (NH), 10 nM 17β-estradiol (E2), 100 nM 4-hydroxytamoxifen (4HT) or 100 nM ICI 182,780 (ICI). Values are the average ± SEM of three independent experiments standardized to the luciferase data obtained in the presence of estrogen and absence of transfected SRA expression vectors.

Figure 5: Coactivation of the ERα AF-1 domain by SRA. **A)** SRA coactivation of GAL-ABα and GAL-ABβ chimeric proteins by SRA. HeLa cells were cotransfected with 100 ng of pBIND (GAL), pBIND-ABα (GAL-ABα) or pBIND-ABβ (GAL-ABβ) and 1000 ng pG5-Luc in the presence or absence of 1000 ng pSCT-SRA. Values are the average ± SEM of four independent experiments standardized to the luciferase data obtained for the GAL4 DNA binding domain in the absence of transfected SRA expression vectors. **B)** SRA coactivation of an AF-2 mutant of ERα. HeLa cells were cotransfected with 250 ng of the $pRST_7$ expression vector for wild type, 3x or TAF2-3x forms of ERα or parent vector alone along with 1500 ng of ERE-E1b-Luc in the presence or absence of 1000 ng of SCT-SRA and treated with ethanol (NH) or 1 nM 17β-estradiol (E2). Values are the average ± SEM of five independent experiments standardized to the luciferase data obtained for wild type ERα in the presence of estrogen and absence of transfected SRA expression vectors.

Figure 6: Coactivation of the ERα AF-1 domain by simultaneous coexpression of SRA and SRC-1e. HeLa cells were cotransfected with 40 ng of pBIND (GAL), pBIND-ABα (GAL-ABα) or pBIND-ABβ (GAL-ABβ) and 1500 ng pG5-Luc in the presence or absence of 1000 ng pSCT-SRA and 1000 ng pCR3.1-SRC-1e. Values are the average ± SEM of five independent experiments standardized to the luciferase data obtained for the GAL4 DNA binding domain in the absence of transfected expression vectors for coactivators.

Figure 7: Activation of amino-terminal deletion mutants of ERα and ERβ by SRA in HeLa cells. **(A)** Forty ng of the $pCMV_5$ expression vectors for ERα and ERα-87C (n=3), or **(B)** pCR3.1 expression vectors for ERα, ERα-109C and ERα-179C (n=3), or **(C)** pCXN2-ERβ and pCMV5-ERβ-46C (n=3) or **(D)** pCR3.1 expression vectors for wild type ERβ and ERβ-143C (n=4) were cotransfected with 1500 ng of ERE-E1b-Luc in the presence or absence of 1000 ng of SCT-SRA and treated with ethanol (NH) or 1 nM 17β-estradiol (E2). Values are the average ± SEM of the indicated number of independent experiments standardized to the luciferase data obtained for wild type receptor in the presence of estrogen and absence of transfected SRA expression vectors.

Figure 8: Simultaneous coactivation of ERα and ERβ by SRA and SRC-1e. HeLa cells were cotransfected with 40 ng of pCR3.1-hERα **(A)** or pCXN2-ERβ **(B)** and 1500 ng of ERE-E1b-Luc in the presence or absence of 1000 ng pSCT-SRA and 1000 ng pCR3.1-SRC-1e and treated

with ethanol (NH) or 1 nM 17β-estradiol (E2). Values are the average ± SEM of five independent experiments standardized to the luciferase data obtained for either receptor in the absence of exogenous coactivator expression.